COORDINATION OF DNA DAMAGE CHECKPOINT SIGNALING AND REPAIR IN *SACCHAROMYCES CEREVISIAE*

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Patrice Yapo Ohouo

August 2013

Coordination of DNA Damage Checkpoint (DDC) Signaling and Repair in *Saccharomyces* Cerevisiae

Patrice Yapo Ohouo, Ph. D.

Cornell University 2013

The DNA damage checkpoint (DDC) is an evolutionarily conserved phosphorylation-based signaling network that maintains genomic integrity. In the budding yeast, *Saccharomyces cerevisiae*, the Mec1 kinase plays a central role in the initiation of the DDC signaling network that ultimately regulates a number of cellular processes including transcription, cell cycle progression and DNA repair. Despite their importance, the mechanisms by which Mec1 signaling controls the DNA repair machinery are not clearly understood. Investigating the functional links between Mec1 signaling and repair factors was of particular interest. I decided to focus on the Rtt107 and Slx4 scaffolding proteins, which are direct targets of Mec1. Rtt107 and Slx4 form a stable complex and also bind to several proteins with established roles in repair. The specific goal of my thesis research was to elucidate the role of Mec1-dependent phosphorylation of Rtt107 and Slx4.

In the first part of my dissertation, I demonstrate that Mec1-dependent phosphorylation mediates a key interaction between the Rtt107-Slx4 complex and the checkpoint activator protein Dpb11. Based on these results I proposed that Dpb11 might represent an anchor point for the recruitment of repair factors bound to Rtt107 and /or Slx4 to sites of damage in a Mec1-dependent manner.

This work also underscored the scaffolding functions of Rtt107 and Slx4, as we were able to expand their interaction network to include the Smc5/6 structural maintenance of chromosome complex and Dpb11.

In the second part of my dissertation I demonstrate an unexpected role for Rtt107 and Slx4 in modulating DDC signaling. I show that by recruiting Rtt107 and Slx4 to Dpb11, Mec1 restricts the access of the Rad9 checkpoint adaptor to Dpb11, thus down regulates the activity of the downstream DDC kinase Rad53.

In conclusion, based on our genetic, biochemical, and proteomic results, I propose that by monitoring and modulating DDC signaling as well as controlling the action of DNA repair enzymes, the Slx4-Rtt107 complex is a crucial coordinator of DDC signaling and DNA repair.

Biographical Sketch of Patrice Yapo Ohouo

Patrice Yapo Ohouo was born in Abidjan, the economical capital of the republic of Cote d'Ivoire in West Africa. After completing high school, Patrice immigrated to the United States of America in 2000 to pursue his undergraduate studies in Biology. Prior to completing his Bachelor of Science in Biology at the State University of New York (SUNY) at Albany, He joined Dr. Igor Roninson's group at the Ordway research institute in Albany, New York. There, under the supervision of Dr. Michael Shtutman he participated in a study aimed at investigating the role of the L1 cell adhesion molecule in breast cancer cell metastasis. Following his graduation from SUNY at Albany, Patrice joined a team led by both Dr. Shtutman and Dr. Ralph Buttyan where as a laboratory technician he developed a set of reporter systems aimed at investigating androgen-mediated gene expression in prostate cancer cells. In 2008, Patrice accepted an offer from the Biochemistry Molecular and Cell Biology program at Cornell University and started his Ph.D. study. He eventually joined Dr. Marcus Smolka's laboratory in 2009 and began work on his dissertation project, which consisted in investigating the regulatory role of DNA damage checkpoint (DDC) signaling in DNA repair. He published his initial work on how the apical DDC kinase Mec1 (human ATR) regulates the replication stress response via phosphorylation of DNA repair factors Rtt107 and Slx4 (*Molecular Cell*, 2010). He also published a follow up work, which reveals a novel function for Rtt107 and Slx4 in the modulation of DDC signaling (*Nature,* 2013).

This work is dedicated to my late father Ohouo Chonou Christian.

ACKNOWLEDGEMENTS

This dissertation symbolizes a milestone in my scientific career. I most certainly subscribe to the African proverb: it takes a whole village to raise a child. My parents, siblings, friends, teachers and colleagues have all contributed to the current wealth of knowledge I have acquired. This dissertation is in many ways the expression of my gratitude.

I would like to begin by thanking my Lord Jesus Christ. Throughout the years, he has blessed my family and me with good health, wisdom and his comforting presence. With him I know all things are possible.

I would like to thank my parents, Ohouo Chonou Christian and Assi Abodin Helene. The life journey of this son and this daughter of farmers who overcame, continues to be a true inspiration to me. I keep their teaching about Love, God and hard work close to my heart, each and everyday.

I want to also give special thanks to my better half and best friend Claudia Reyes. She is my true motivation. For more than four years she has been away from me, working hard toward her dream of becoming a Doctor of dentistry while raising our beautiful daughter Maya. Her attitude and determination is what pushed me. How could I complain about long days in the lab or the cold Ithaca weather? We did face difficult moments along the way but at the end of each and every day we were there for each other. Today we are stronger for it in so many aspects of our lives.

I would like to extend special thanks to my mentor Dr. Marcus Smolka. It has been a remarkable and truly enjoyable experience being Marcus' first

graduate student and I am so proud. Marcus has taught me a lot about science but I have learned a great deal from watching him manage the challenges set in front of a new and aspiring young professor. I especially value the patience and open mind he kept when discussing intricate biological problems. I intend to rely on this attitude as I move toward the scientific pinnacles I plan to reach.

I also want to express my deep and sincere appreciation for Dr. Francisco Bastos de Oliveira who has been a friend, a mentor and a big brother to me. It was a pleasure working with him. When facing long hours in the Lab Francisco's hard work and ability to set a friendly and warm Lab atmosphere were both encouraging and comforting.

To end, I would like to thank Dr. Lana Slalov and Dr. John Broome for their kind words of advice and support over the years and their special attention to my scientific career.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Author's Contribution

Chapter 2, in part, material as it appears in Mol Cell 2010; 39:300-6. 5. **Ohouo P**, Bastos de Oliveira F, Almeida B, Smolka M. DNA damage signaling recruits the Rtt107-Slx4 scaffolds via Dpb11 to mediate replication stress response. The dissertation author was the primary investigator and co-first author of this paper. In the paper listed above, Patrice Y. Ohouo and Marcus Smolka carried out the Mass spectrometry-based proteomic analysis that lead to the identification of Dpb11 as a binding partner of Rtt107 and Slx4. Francisco Bastos de Oliveira generated the mutant allele of SLX4 used to show the importance of the Rtt107-Slx4-Dpb11 complex in promoting cellular viability in stress condition. The other co-first author, Francisco Bastos de Oliveira generated the mutant allele of SLX4 used to show the importance of the Rtt107-Slx4-Dpb11 complex in promoting cellular viability in stress condition (Figure 2-4).

Chapter 3, in part, material as it appears in Nature*, 493(7430)*, 120-124. **Ohouo PY**, Bastos de Oliveira FM, Liu Y, Ma CJ, Smolka MB. DNA-repair scaffolds dampen checkpoint signalling by counteracting the adaptor Rad9. The dissertation author was the primary investigator and first author of this paper. In the paper listed above, Patrice Y. Ohouo and Marcus Smolka carried out the Mass spectrometry-based proteomic analysis showing that the hyperactivation of DNA damage checkpoint signaling occurs at the level of Rad53. Yi Liu performed the Mass spectrometry analysis on phosphorylated Slx4, which hinted to the importance of phosphorylated Serine 486 (S486) of Slx4 in the Slx4-Dpb11 interaction. Yi Lui also performed the pull down experiments using the recombinant BRCT domains

of Dpb11 showing that both Slx4 and Rad9 bind the same domain in Dpb11 (Figure 3-2). Patrice Ohouo performed the genetic analysis showing that the Rad1 and Dpb11-dependent functions of Slx4 are distinct from each other. Patrice Y. Ohouo performed the Slx4 over-expression experiment indicating that Slx4 competes with Rad9 for Dpb11 binding. Francisco Bastos de Oliveira generated the mutant allele of SLX4 used to show the importance of S486 in the Slx4-Dpb11 interaction (Figure 3-2).

Chapter 1

INTRODUCTION

DNA damage constantly challenges genomic integrity, which is critical for proper cellular proliferation. Environmental agents such as ultraviolet light and cigarette smoke but also key biological processes such as transcription and DNA replication induce the formation of lesions and breaks that damage DNA. In turns, DNA damage promotes mutation, deletion and chromosomal translocation events, which undermines the integrity of genomes leading to genomic instability, a hallmark of cancers (Hoeijmakers, 2009; Phillips, Hewer, Martin, Garner, & King, 1988). Genomic instability represents a critical link between DNA damage and tumorigenesis. It contributes to the deregulation of checkpoint and cell death mechanisms that otherwise circumscribe growth in normal cells (Lengauer, Kinzler, & Vogelstein, 1998; Lowe, Cepero, & Evan, 2004). This is illustrated by fact that genomic instability confers cancer cells their significant proliferative advantage by promoting the activation of proto-oncogenes or the deactivation of tumor suppressor genes (Beckman & Loeb, 2006; Lowe et al., 2004). Understanding the cellular mechanisms responsible for maintaining genomic integrity when DNA damage occurs is therefore critical to our ability to prevent and effectively combat cancers. My graduate work focused on a major genome maintenance mechanism called the replication checkpoint. This highly conserved pathway is a branch of the DNA damage checkpoint pathway that monitors the integrity of the genome during DNA replication (B. A. Desany, A. A. Alcasabas, J. B. Bachant, & S. J. Elledge, 1998; Foiani et al., 2000; Lopes et al., 2001; K Myung & Kolodner, 2002).

DNA replication, although essential, is a highly dynamic process that can inadvertently compromise genomic integrity (K. Myung, Datta, & Kolodner, 2001; J. Tercero, Longhese, & Diffley, 2003). When damage occurs during DNA replication, the replication checkpoint machinery quickly assembles at the site of damage where it mediates a phosphorylation based signaling cascade (S. Chen & Zhou, 2009; Friedel, Pike, & Gasser, 2009; Santocanale & Diffley, 1998). In humans and in the budding yeast *Saccharomyces cerevisiae* respectively, ATR and Mec1 are the apical or so-called sensor kinases that initiate the phosphorylation cascade at the center of the replication checkpoint pathway (Cortez, Guntuku, Qin, & Elledge, 2001; Friedel et al., 2009). This phosphorylation cascade targets a plethora of effector proteins to regulate key cellular processes such as transcription; cell cycle progression and DNA repair in order to promote genomic integrity (J. Cobb, Bjergbaek, Shimada, Frei, & Gasser, 2003; J. A. Cobb et al., 2005; B. Desany, A. Alcasabas, J. Bachant, & S. Elledge, 1998; Lopes et al., 2001; Tourrière, Versini, Cordón-Preciado, Alabert, & Pasero, 2005). The important role of this pathway in preserving genomic integrity is illustrated by the fact that mutations in its components lead to increased genomic instability and predisposition to cancers (K. Myung et al., 2001). Despite the well-established importance, exactly how the phosphorylation events mediated by the replication checkpoint pathway maintain genomic integrity is not completely understood. My dissertation project focused on the Mec1 kinase. I wanted to elucidate the mechanistic roles of Mec1-dependent phosphorylation in DNA repair. In the first part of my work (chapter 2) I show that Mec1 phosphorylates key scaffolding proteins involved in DNA repair in order to mediate protein interactions

important for viability in the presence of DNA damaging agents. In the second part of my work (chapter 3), I present data suggesting that Mec1-dependent phosphorylation enables the same repair proteins to play direct roles in the modulation of replication checkpoint signaling.

First in this introduction, I will present some of the key concepts and well accepted models that were central to my project.

DNA replication as a source of genomic instability

DNA replication is a highly dynamic process that has been shown by several studies to render the genome highly susceptible to damage (Katou et al., 2003; Lopes et al., 2001; J. A. Tercero & Diffley, 2001). DNA replication is mediated by an extensive network of proteins (replisome), which unwinds and replicates the parental strands of DNA to form what is known as replication forks. The high susceptible for DNA to become damaged during the replication process is due in part to the likelihood of an encounter between forks and DNA lesions or stable protein-DNA complexes. Such encounters can lead to fork stalling, which can eventually promotes the dissociation of the replisome, also known as fork collapse (Azvolinsky, Giresi, Lieb, & Zakian, 2009; Sancar, Lindsey-Boltz, Unsal-Kaçmaz, & Linn, 2004). Upon collapse, the exposed fork structures can be further processed into double strand breaks (DSBs) by fork structure specific nucleases (Costanzo et al., 2003). Deregulated or faulty DNA replication is also linked to increased genomic instability. DNA replication is tightly regulated in space and time in order to ensure proper coordination with cell cycle progression. Cyclins play critical roles in this regulation. In the budding yeast, aberrant expression of cyclins leads to faulty DNA

replication events, which were shown to induce DSBs (Lengronne & Schwob, 2002; Tanaka & Diffley, 2002). Furthermore, the overexpression of oncogenes such as c-Myc, Cyclin E and Cdc45 found in several cancers is suggested to contribute to genomic instability by inducing untimely initiation of DNA replication(Bartkova et al., 2005; Ekholm-Reed et al., 2004; Spruck, Won, & Reed, 1999; Vafa et al., 2002).

In order to counter the detrimental effects of DNA replication and preserve the integrity of the genome, cells have evolved a highly conserved surveillance mechanism called the replication checkpoint (See figure 1.1).

The replication checkpoint is a barrier to genomic instability and cancer

The replication checkpoint pathway is critical to the preservation of genomic integrity. In yeast, the important contribution of the replication checkpoint pathway to genomic stability is illustrated by the fact that deletion of components such as Mec1 leads to gross chromosomal rearrangements (GCR) such as deletion of chromosome arms, addition of telomeres and translocation events (K. Myung et al., 2001). More importantly, these rearrangements occur in the absence of replication stress inducing drugs, further supporting the idea that DNA damage commonly occurs during DNA replication. In humans, mutations in the gene encoding ATR are associated with congenital defects and cancer predisposition syndromes (Bartek, Bartkova, & Lukas, 2007; Gorgoulis et al., 2005). Moreover, ATR deficient cells display increased chromatid breaks *in vitro* (Brown & Baltimore, 2003). Due to its importance, several studies conducted over the course of the last two decades have focused understanding the molecular mechanisms that underpin the action of the

replication checkpoint machinery, especially how the machinery becomes assembled.

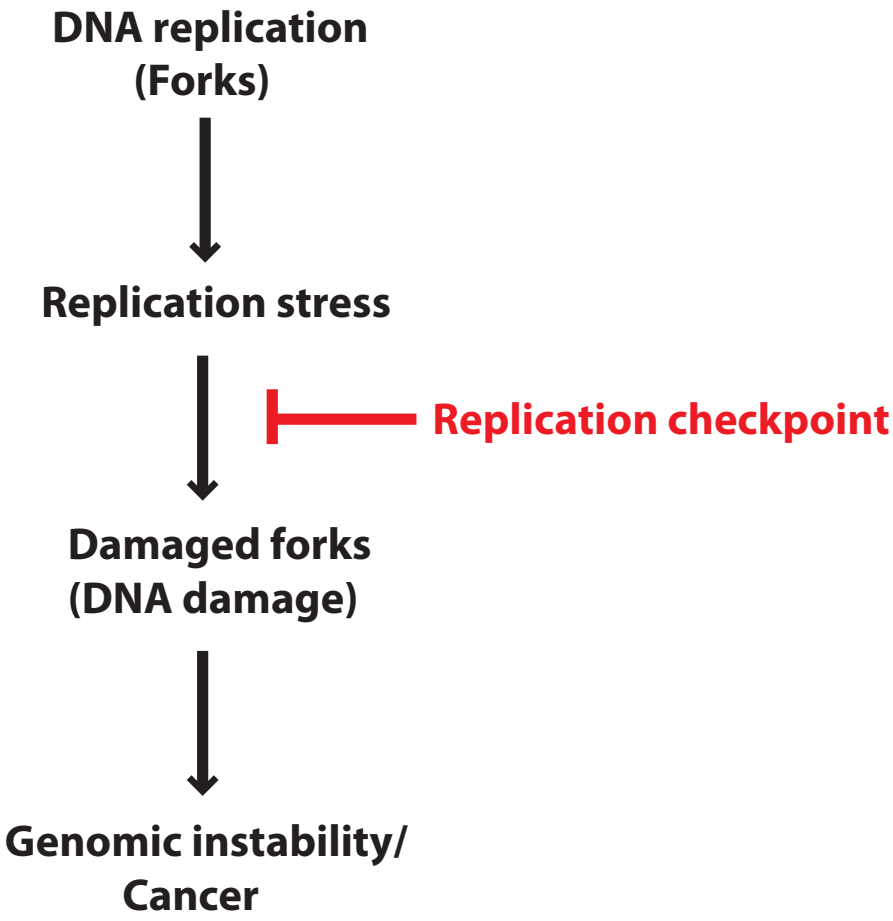

DNA replication
(Forks)
Replication stress
Replication checkpoint
Damaged forks
(DNA damage)
Genomic instability/
Cancer

Figure 1-1

Figure 1.1. The replication checkpoint preserves genomic integrity during DNA replication. Replication stress bought on by blockages that impede the progression of forks can induce DNA damage by destabilizing replication forks. Damaged forks, which represent a source of DNA damage, promote genomic instability and induce tumorigenesis. The replication checkpoint promotes fork stability and thus preserves genomic integrity in the presence of replication stress.

Replication checkpoint signaling in budding yeast

The budding yeast was instrumental in deciphering key mechanistic aspects of the replication checkpoint pathway. The high level of conservation of the pathway between yeast and humans added to the capability to delete Mec1 (enabled by the deletion of the SML1 gene that encodes a ribonucleotide reductase inhibitor (X. Zhao, Muller, & Rothstein, 1998)), which in humans leads to lethality, makes the budding yeast perfectly suited for the study of the replication checkpoint pathway (Cortez et al., 2001; Craven, Greenwell, Dominska, & Petes, 2002; X. Zhao et al., 1998). Genetic and biochemical studies conducted in this model organism have help expose key components of the extensive network of protein interactions that make up the replication checkpoint pathway. This interaction network is composed of kinases, DNA binding and adaptor proteins that assemble on DNA.

Single strand DNA (ssDNA) is the primary signal for the assembly of the replication checkpoint machinery. SsDNA occurs only transiently in the cell as it is rapidly coated by the ssDNA binding complex RPA (Wold, 1997). There is a limited amount of RPA coated ssDNA at unchallenged forks, which results the unwinding of the parental double stranded DNA. However, during replication stress, the stalling of the replicative polymerases leads to their uncoupling from the helicase complex and results in the exposure of extensive amounts of ssDNA-RPA complex (Majka, Binz, Wold, & Burgers, 2006; Wold, 1997; Zou & Elledge, 2003). SsDNA can Alternatively, be generated via resection through the action of nucleases involved in DNA repair pathways (Majka et al., 2006; Wold, 1997; Zhu, Chung, Shim, Lee, & Ira, 2008). The accumulation of the ssDNA-RPA complex, is recognized by Mec1, which becomes

recruited via its cofactor, the Ddc2 (human ATRIP) protein (Rouse & Jackson, 2000). Following its recruitment, the kinase activity of Mec1 needs to be stimulated several folds in order to initiate checkpoint signaling. The Dpb11 (human TOPBP1), Ddc1 (human RAD9) and more recently Dna2 proteins were shown to play critical roles in the activation of Mec1. These potent activators of Mec1 all contain key aromatic residues in their unstructured carboxy termini that mediate their Mec1 activating functions (Kumar & Burgers, 2013; V. Navadgi-Patil & Burgers, 2008; V. M. Navadgi-Patil & Burgers, 2009). Once activated, Mec1 phosphorylates a key downstream effector protein that is the Rad53 kinase. This phosphorylation is essential for the activation of Rad53. It is suggested to act as a priming activation event, which is spread via autophosphorylation amongst other Rad53 molecules recruited at the site of damage (See figure 1-2) (Lee, Schwartz, Duong, & Stern, 2003; Liao et al., 2000; M. F. Schwartz et al., 2002; Vialard, Gilbert, Green, & Lowndes, 1998).

Adaptor proteins are an important component of the replication checkpoint pathway. They ensure the co-localization of the different components of the machinery. For instance, the recruitment of Rad53 molecules near Mec1, which is required for the efficient transduction of signal from Mec1 to Rad53 is mediated by either one of two adaptor proteins that are Rad9 (human 53BP1) and the replication fork component Mrc1 (human Claspin) (Alcasabas et al., 2001; S. Chen & Zhou, 2009; Gilbert, Green, & Lowndes, 2001; M. F. Schwartz et al., 2002; Sun, Hsiao, Fay, & Stern, 1998). The extensive phosphorylation of these adaptors by Mec1 enables their recognition and binding via the fork head associated (FHA1 and FHA2) domains of Rad53 (M. F. Schwartz et al., 2002; Smolka et al., 2006). It is important to

point out the differences that exist between these adaptor proteins. Rad9 helps recruit Rad53 at double strand breaks or at ssDNA gaps behind forks (See Figure 1-2) while Mrc1 promotes Rad53 recruitment and activation at stalled forks (S. Chen & Zhou, 2009). As a result, the adaptor function of Mrc1 is limited to S phase as opposed to Rad9, which can mediate Rad53 activation through out the different phases of the cell cycle (Hammet, Magill, Heierhorst, & Jackson, 2007; Katou et al., 2003; Naylor, Li, Osborn, & Elledge, 2009; Wysocki et al., 2005).

Next I will discuss two important components of the replication checkpoint machinery that help promote efficient signaling.

The roles of the 9-1-1 complex and Dpb11 in replication checkpoint signaling

As mentioned above, Ddc1 and Dpb11 are important stimulator for the kinases activity of Mec1. However, their roles in checkpoint signaling are multi-faceted and rather complex.

Ddc1 is a key component of the budding yeast heterotrimeric known as the 9-1-1 complex. The complex is named after the Rad9, Rad1 and Hus1 human components. They correspond respectively to Ddc1, Mec3 and Rad17 in budding yeast (Ellison & Stillman, 2003; V. M. Navadgi-Patil & Burgers, 2009). The 9-1-1 complex is a clamp that is structurally related to the proliferating cell nuclear antigen (PCNA) complex, which functions as a processivity factor during DNA replication. The 9-1-1 checkpoint clamp is loaded on the 5' single strand-double strand DNA junction in an ATP dependent manner by the heteropentameric Rad24-RFC complex, which is one of the three RFC-like clamp loaders known to date (Ellison & Stillman, 2003). The importance of the 9-1-1 complex in checkpoint

signaling is illustrated by the fact that deletion of any of its components abrogates the DNA damage checkpoint (Kondo, Matsumoto, & Sugimoto, 1999; Marchetti et al., 2002). Studies have revealed that the 9-1-1 complex promotes checkpoint signaling by stimulating the activity of Mec1 but also by helping recruit Dpb11, which interacts with its Ddc1 component (see Figure 1-2) (Pfander & Diffley, 2011).

Dpb11 is an essential multi BRCT containing protein that plays roles in the initiation of DNA replication as well as checkpoint signaling. In the context of DNA replication, Dpb11 is essential for the assembly of the replication machinery, where it forms a stable complex with the initiator proteins Sld2 and Sld3 that have been previously phosphorylated by CDK (Masumoto, Muramatsu, Kamimura, & Araki, 2002; Tak, Tanaka, Endo, Kamimura, & Araki, 2006; Zegerman & Diffley, 2010). Dpb11 also was shown to helps load the replicative polymerase Pol ε onto the pre-replicative complex (Masumoto, Sugino, & Araki, 2000). Though Dpb11 plays critical roles during the initiation of DNA replication, it does not appear to be an integral component of moving forks (Gambus et al., 2006; Takayama et al., 2003). Its function and localization at damaged forks point to a re-recruitment mechanism at damaged forks where it is involved in checkpoint signaling. There are two main aspects to the roles of Dpb11 roles in checkpoint activation. Dpb11 promotes the activation of Mec1 via its C terminus and help activate Rad53 by recruiting of Rad9 near Mec1 (see Figure 1-2) (Pfander & Diffley, 2011). CDK-dependent phosphorylation of Rad9 is required for the interaction between Rad9 and Dpb11. The latter recognizes and binds to CDK phosphorylated motifs on Rad9 via its N-

terminal BRCT domains 1 and 2 (Pfander & Diffley, 2011; G. Wang, Tong, Weng, & Zhou, 2012).

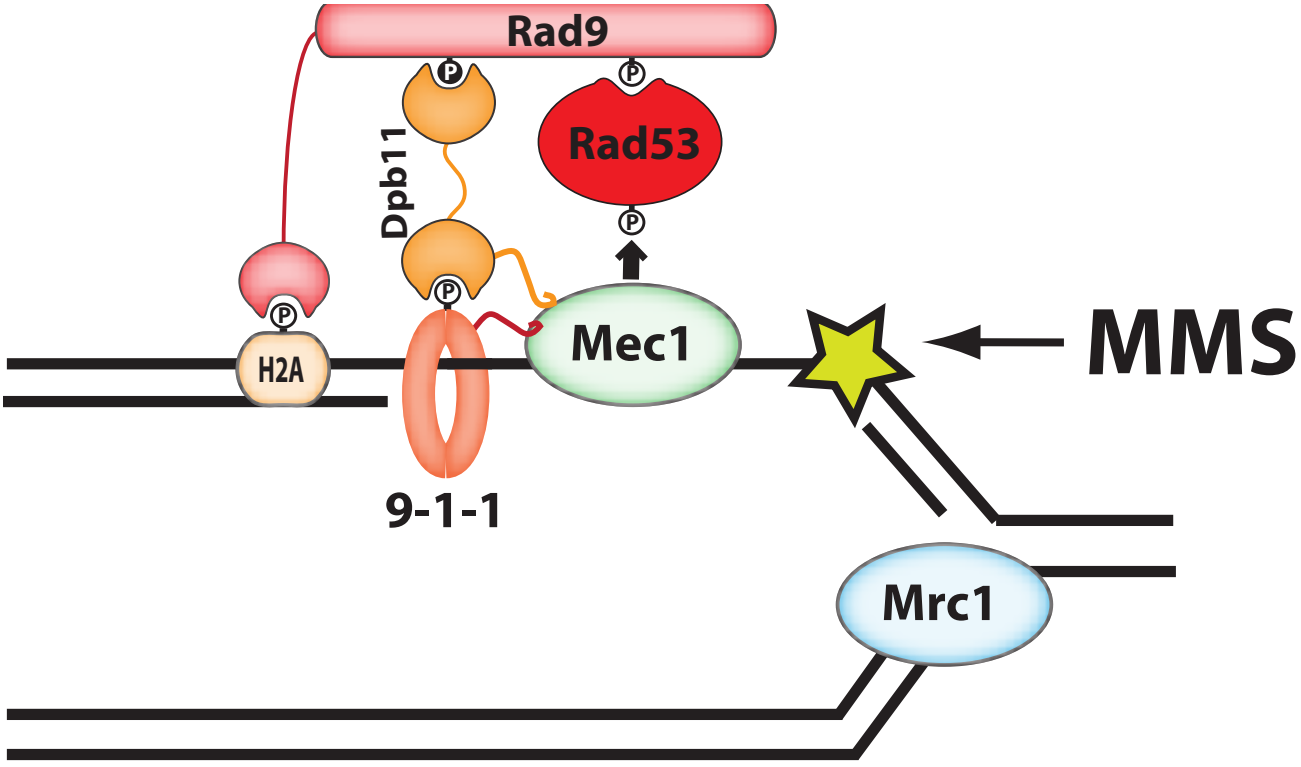


Figure 1-2

Figure 1.2. Rad9-dependent Rad53 activation at lesions behind forks. MMS induced DNA alkylation leads to the stalling of the DNA replicative polymerases and accumulation of ssDNA behind the fork. The RPA-ssDNA complex enables the recruitment of Mec1. The 9-1-1 complex is recruited at the 5' ssDNA-dsDNA junction where it promotes the recruitment of Dpb11. H2A recruited near the site of damage is phosphorylated by Mec1 and mediates the recruitment of Rad9. Mec1 phosphorylates Rad9 at the S/TQ cluster (SCD) domain, creating a docking site for Rad53 molecules, which are recruited via their FHA domains. Rad9 bound Rad53 molecules are activated by an initial trans-phosphorylation event by Mec1 followed by autophosphorylation of Rad53 molecules. The Mrc1 adapator indicates the relative position of the fork.

Beyond the mechanisms regulating the assembly and activation of the replication checkpoint, lays the functional roles of this pathway. Studies have established the links between Mec1 and Rad53-depedent phosphorylation of several proteins and the regulation of a plethora of cellular processes (Smolka, Albuquerque, Chen, & Zhou, 2007) including replication fork stability, transcription, cell cycle progression and DNA repair (Huang, Zhou, & Elledge, 1998; Labib & De Piccoli, 2011) (See Figure 1-3 for summary). For instance, Mec1-dependent phosphorylation of the H2A histone variant is suggested to contribute to repair as phosphorylated H2A (γH2A) helps recruit chromatin remodelers and repair proteins to sites of damage (Bassing et al., 2002; Celeste et al., 2002; Huen et al., 2007; X. Wang, Andreassen, & D'Andrea, 2004). The replication checkpoint has also been shown to regulate transcription. A well-known example is the regulation of the transcription of the RNR genes, which are involved in dNTP synthesis (Chabes et al., 2003). There, Rad53 relieves the inhibitory role of the Crt1 repressor on the transcription of the RNR genes by phosphorylating and activating the Dun1 kinase (Huang et al., 1998).

Next, I will discuss in detail how Mec1 and Rad53 were shown to promote replication fork stability and regulate cell cycle progression.

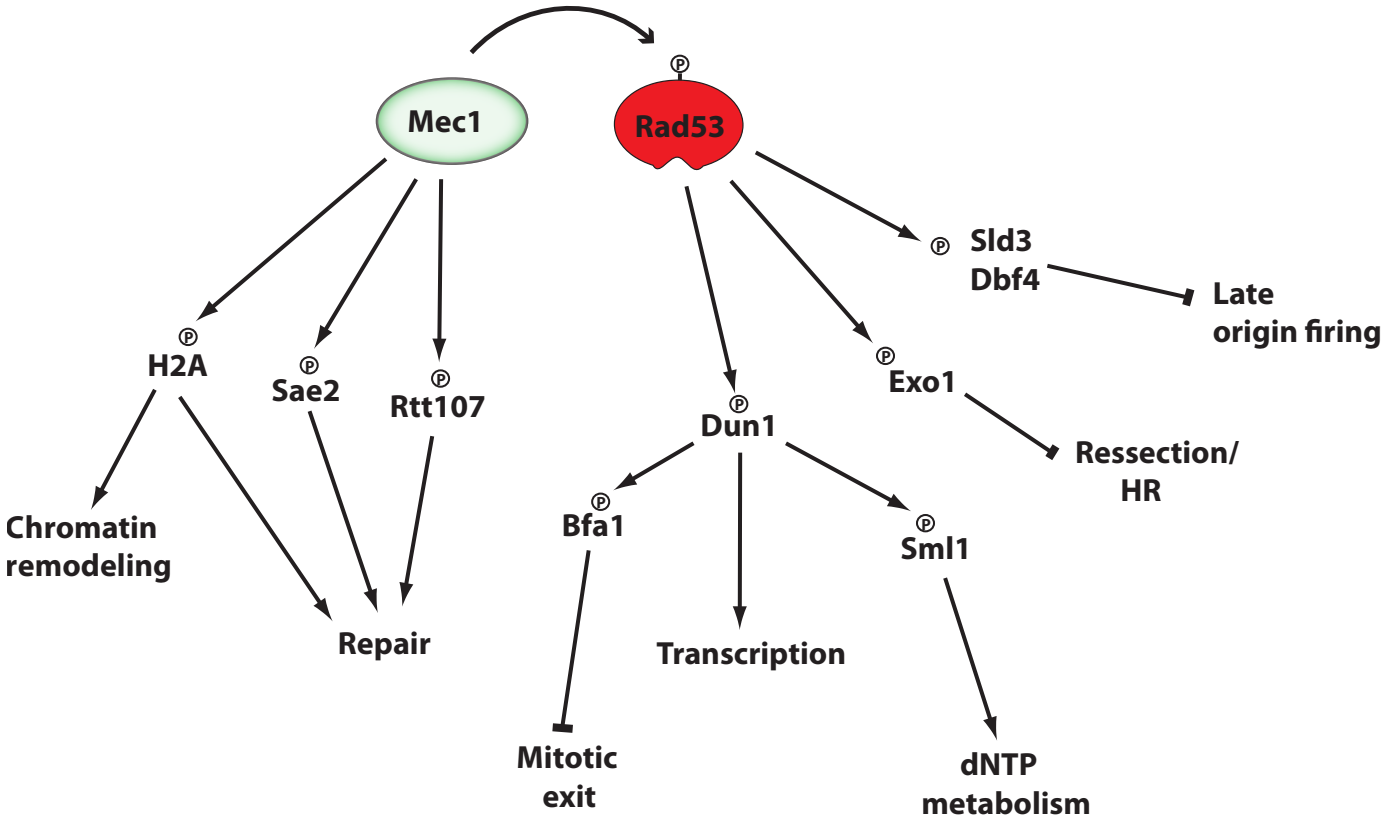

Mec1
Rad53
H2A
Sae2
Rtt107
Sld3
Dbf4
Late
origin firing
Exo1
Ressection/
HR
Dun1
Bfa1
Sml1
Chromatin
remodeling
Repair
Transcription
Mitotic
exit
dNTP
metabolism

Figure 1-3

Figure 1.3. The kinase activity of Mec1 and Rad53 regulates several cellular processes. The arrowheads indicate the inhibitory or stimulating action of the kinases via the phosphorylation of the indicated targets. Mec1-dependent phosphorylation of H2A recruits the chromatin remodeler INO80 and promotes repair (Thiriet & Hayes, 2005; van Attikum & Gasser, 2009). Mec1 is suggested to regulate the repair function of Sae2 and Rtt107 (Baroni, Viscardi, Cartagena-Lirola, Lucchini, & Longhese, 2004; Flott et al., 2007; Rouse, 2004). Rad53 activates Dun1 to regulate transcription and Sml1 activity (Allen, Zhou, Siede, Friedberg, & Elledge, 1994; Huang et al., 1998; X. Zhao & Rothstein, 2002). Rad53 is implicated in inhibition of mitotic exit via Dun1 dependent phosphorylation of Bfa1 (Liang & Wang, 2007). Rad53 inhibits the roles of Exo1 and Sld3 in resection and late origin firing respectively (Morin et al., 2008; Zegerman & Diffley, 2010).

Roles of Mec1 and Rad53-dependent phosphorylation at replication forks

DNA Lesions and protein-DNA complexes induce replication stress that was shown to have a direct effect on fork progression and stability (J. A. Tercero & Diffley, 2001). The DNA replication induced gross chromosomal rearrangements observed in checkpoint defective cells indicate a role for Mec1 and Rad53 in the stabilization of replication forks (K. Myung et al., 2001). These kinases are suggested to play important roles in maintaining forks in a replication competent state during replication stress. This is supported by the fact that *mec1Δ* or *rad53Δ* cells are unable to complete DNA replication during or even after the removal of stress (B. A. Desany et al., 1998; Lopes et al., 2001; Sogo, Lopes, & Foiani, 2002; J. A. Tercero & Diffley, 2001). Mec1 and Rad53 phosphorylate several components of the DNA replication machinery (Matsuoka et al., 2007; Smolka et al., 2007). Studies investigating the mechanistic roles of such phosphorylation have revealed that checkpoint signaling directly contributes to fork stability by stimulating dNTP metabolism, maintaining the components of the replication machinery on stalled forks and preventing excessive resection.

The fact that the otherwise essential MEC1 and RAD53 genes become dispensable when the pools of dNTPs are stabilized by the deletion of the gene encoding the Sml1 ribonucleotide reductase inhibitor suggests that dNTP metabolism is a critical function of these kinases (X. Zhao et al., 1998; X. Zhao & Rothstein, 2002; Zheng et al., 1993). Limiting amounts dNTP's can induce replication stress by promoting the stalling of the replicative polymerases thus altering the organization of the replisome. This is illustrated by the increase amount

of ssDNA at forks upon exposure to the dNTP depleting agent hydroxyurea (HU) (De Piccoli et al., 2012; Sogo et al., 2002). Replication checkpoint defective cells display remarkable abnormalities under such conditions. For instance, *rad53Δ* cells treated with HU accumulate aberrant fork structures that appear to result from the reversal of forks (Sogo et al., 2002). Overall, these observations suggest that the stimulation of dNTP production by Rad53 is indeed critical for fork stability during replication stress.

The stalling of forks by depletion of dNTP's or other means is suggested to lead to the dissociation of the replication machinery from the fork structures. Replication checkpoint signaling appears to play important roles in counteracting such events (Sogo et al., 2002). Initial studies conducted in the presence of HU showed that replication stress in the absence of Mec1 and Rad53 promotes the accumulation of ssDNA at origins of replication, indicating the dissociation of the replisome from the DNA structures (J. A. Cobb et al., 2005; Cotta-Ramusino et al., 2005; Katou et al., 2003; Lucca et al., 2004; Raveendranathan et al., 2006). This view is now being challenged by recent studies showing that in the absence of replication checkpoint kinases, although uncoupled, the helicase and polymerases are still bound to the replication fork structures (De Piccoli et al., 2012; Luciani, Oehlmann, & Blow, 2004). Moreover, the Labib group recently showed that instead of the dissociation of the replication machinery from forks, the deletion of Mec1 or Rad53 lead to the simultaneous firing of early and late origins. While this observation is not new, the same study also revealed a difference in Mec1-depedent phosphorylation of components of the replisome in the presence or absence of Rad53 (De Piccoli et

al., 2012). These recent observations suggest that the checkpoint kinases may regulate replication fork function rather than stability.

Another aspect of the role of replication checkpoint signaling on fork stability is the counteraction of nuclease activity mediated, by the Exo1 exonuclease at stalled forks (Morin et al., 2008). The deletion of EXO1 reduces the sensitivity of *rad53Δ* cells treated with the DNA alkylating agent methyl methanesulfonate (MMS) and appears to suppress defects in fork progression (Segurado & Diffley, 2008). As a result, Rad53 is suggested to promote fork integrity under replication stress conditions by phosphorylating and inhibiting excessive Exo1 mediated resection of stalled forks.

As mentioned above, the roles of the replication checkpoint signaling extend beyond the scope of the fork and affect other key biological processes, including the regulation of cell cycle progression.

Replication checkpoint signaling and the regulation cell cycle progression

Perhaps the most striking aspect of the replication checkpoint signaling is the regulation of cell cycle progression. In fact, it is this feature that led to the identification of Rad9 as the first DNA damage checkpoint protein (T. A. Weinert & Hartwell, 1988). Rad9 protein contains several domains, which mediate its recruitment to chromatin or to sites of damage. The TUDOR and BRCA1 C-terminus homology (BRCT) domain of Rad9 mediate its chromatin recruitment by binding respectively to Dot1 methylated lysine 79 of histone H3 (H3K79) and to histone H2A phosphorylated at serine 129 (S129) by Mec1 (Usui, Foster, & Petrini, 2009). Rad9 also contains clusters of Cdc28 (CDK) phosphorylation sites that mediate its

recruitment to sites of damage when recognized and bound by the BRCT domains of Dpb11 (Granata et al., 2010; Pfander & Diffley, 2011; G. Wang et al., 2012). Another key feature of Rad9 is the S/QT cluster domain (SCD), which when phosphorylated by Mec1, is recognized by either one of the two fork head associated (FHA) domains of Rad53 (M. Schwartz, Lee, Duong, Eminaga, & Stern). This domain is critical for the ability of Rad9 to activate Rad53 (See Figure 1-4 for schematic representation of the Rad9-Rad53 activating module).

Rad9 is a potent activator of Rad53 and the interaction between these two proteins is critical for Rad9 to play its cell cycle progression regulatory roles. Activated Rad53 is suggested to regulate cell cycle progression via two major pathways. First, it was shown to block late origin firing by phosphorylating the DNA replication initiation protein Sld3 and the regulatory subunit of the Cdc7 kinase, Dbf4 (Lopez-Mosqueda et al., 2010; Zegerman & Diffley, 2010). Second, Rad53 was also shown to inhibit mitotic exit by enabling the GAP complex protein Bfa1 to bind and sequester Tem1, a component of the mitotic exit network (MEN) (Liang & Wang, 2007).

Due to its ability to halt cell cycle progression, there is a clear need to regulate the kinase activity of Rad53. While most studies focused on the activation of Rad53, a few have helped further our understanding of how this kinase can be downregulated.

Taming the kinase activity of Rad53

Rad53 activation appears to be a balancing act. While initial activation of Rad53 is critical for fork stabilization (inhibition of excessive fork resection by the Exo1 endonuclease), hyperactivation of the Rad9-Rad53 pathway can halt cell cycle progression and be detrimental to the cell (T. A. Weinert & Hartwell, 1988). Additionally, the down regulation of Rad53 appears to be important for restarting stalled forks (Szyjka et al., 2008).

The fact that Rad53 mediated cell cycle arrest is reversible hinted at the existence of cellular mechanisms for the downregulation of its kinase activity. In fact, Rad53 deactivation is a prerequisite for cells to escape cell cycle arrest whether DNA damage is persistent or not, which correspond to processes known as adaptation or recovery respectively (van Vugt, Brás, & Medema, 2004; Vidanes et al., 2010). While the molecular mechanisms enabling the adaptation or recovery processes remain poorly understood, several studies point to the involvement of protein phosphatases in the dephosphorylation and consequent deactivation of Rad53. Work from the Diffley group has shown that the pools of phosphorylated Rad53 molecules are dephosphorylated following replication stress. Most strikingly that study showed that de Novo Rad53 protein synthesis was not required for checkpoint activation and recovery (J. Tercero et al., 2003). As a result, phosphatases have been implicated in the deactivation and down regulation of Rad53. The Ptc2, Ptc3 and Pph3 phosphoprotein phosphatases appear to play important roles in the dephosphorylation of Rad53 as cells lacking these phosphatases show persistent Rad53 activation even after the removal of the

replication stress-inducing agent (Leroy et al., 2003; O'Neill et al., 2007). This is further supported by the fact that Ptc2 and Pph3 respectively bind to the FHA1 and kinase domains of Rad53 (Guillemain et al., 2007; Leroy et al., 2003; O'Neill et al., 2007). The function of these phosphatases in down regulating Rad53 does not seem to overlap. In fact, persistent Rad53 activation and defect in recovery are more striking when all three phosphatases are knocked out (Kim, Hicks, Li, Tay, & Haber, 2011). Moreover, in contrast to Ptc2 and Ptc3, Pph3 is suggested to mediate Rad53 deactivation in the presence of double strand break (DSB) (O'Neill et al., 2007). The exact roles of these phophatases remains to be clarified especially when Pph3 was shown to dephosphorylate γH2A, which is upstream of the Rad9-Rad53 pathway (Bazzi, Mantiero, Trovesi, Lucchini, & Longhese, 2010; Keogh et al., 2006). Interestingly, the rapid association and dissociation of Rad53 from damaged forks (Gilbert et al., 2001) does not appear to agree with a model where phosphatases are the sole mediators for the spatio-temporal down regulation of Rad53.

In sum, Rad9 binding to Rad53 promotes a robust activation of Rad53 and underlies Rad9's ability to delay cell cycle progression. This function of Rad9 is suggested to allow time for repair and therefore to be beneficial. In humans the ability of the checkpoint to halt cell cycle progression represent a barrier to tumorigenesis. In fact, the ATR-CHK1 pathway was shown to regulate cell cycle progression by targeting and inhibiting the CDC25A and CDC25C phosphatases, which are required for the G1 to S phase transition and entry in mitosis respectively (M. S. Chen, Ryan, & Piwnica-Worms, 2003; Dai & Grant, 2010). In addition, CHK1 dependent activation of the P53 tumor suppressor has been shown to lead to

growth arrest or apoptosis mediated cell death depending on the extent of the DNA damage (Baker et al., 1989; Baker, Markowitz, Fearon, Willson, & Vogelstein, 1990; Bunz et al., 1998; Polyak, Xia, Zweier, Kinzler, & Vogelstein, 1997).

Coordination of checkpoint signaling and repair

In order to counter the detrimental effects DNA damage has on the genome, cells have to be able to detect the damage, mobilize effector proteins to the site of damage and eventually repair the damage. These are the essential pillars of the DNA damage response (DDR) mechanism (T. Weinert, 1998a, 1998b). In the context of DNA replication, the amount of RPA-coated ssDNA determines the recruitment of Mec1 and Rad53 (Zou & Elledge, 2003). The cell cycle delay promoted by replication checkpoint signaling is an important aspect of the pathway's contribution to the repair process as it allows enough time for repair to take place. This view is supported by the fact that cells lacking components of the pathway progress into mitosis with significant damage and decreased viability (Hartwell & Weinert, 1989; T. A. Weinert & Hartwell, 1988). Additional evidence suggests that the replication checkpoint plays more direct roles in the repair process. In fact, several targets of Mec1 and Rad53 are either repair proteins or key components of the DNA repair machinery (Labib & De Piccoli, 2011; Smolka et al., 2007). For instance, the phosphorylation of H2A by Mec1 on serine 129 (S129) was shown to mediate the recruitment of the INO80 chromatin remodeler complex, which promotes homologous recombination (HR) mediated repair (Thiriet & Hayes, 2005; van Attikum & Gasser, 2009). Phosphorylated H2A also recruits the Rtt107 scaffolding

proteins, which interacts with several proteins involved in repair (Li et al., 2012; P. Y. Ohouo, Bastos de Oliveira, Liu, Ma, & Smolka, 2013). In addition, Mec1 has been suggested to promote the repair functions of the Sae2 and Slx4 repair proteins by regulating associated nuclease activities (Baroni et al., 2004; Flott et al., 2007). Rad53 appears to also play important roles in DNA damage repair. The down regulation of the nuclease activity of Exo1 by Rad53 is proposed to inhibit HR mediated repair and suggest a role for Rad53 in DNA damage repair choice (Alabert, Bianco, & Pasero, 2009; de la Torre-Ruiz & Lowndes, 2000; Morin et al., 2008). While the kinase activities of Mec1 and Rad53 play clear roles in mobilizing effector proteins to damaged forks, several aspects of the roles of Mec1 and Rad53 in the coordination of repair remain to be understood. For example, the fact that Mec1 phosphorylates several effector proteins independently of Rad53 suggests that Mec1 and Rad53 play distinct roles in repair. This hypothesis is further supported by the fact that deletion of EXO1 rescues the MMS sensitivity of *rad53Δ* cells but not that of *mec1Δ* cells (Segurado & Diffley, 2008). Among the direct Mec1 targets figure the Rtt107 and Slx4 scaffolding proteins (Flott & Rouse, 2005; Rouse, 2004). In order to investigate the specific roles of Mec1 in repair and establish a mechanistic link between checkpoint signaling and repair, I decided to study the functional relevance of the Mec1-dependent phosphorylation of Rtt107 and Slx4.

The Rtt107 and Slx4 scaffolding proteins

DNA repair benefits from inputs that expand beyond replication checkpoint signaling. Ubiquitin and sumo ligase activities were also shown to play key roles in

repair (Cremona et al., 2012; Jackson & Durocher, 2013). Scaffolding proteins are central to the coordination of these different inputs, which is critical for efficient repair. In budding yeast, Rtt107 and Slx4 are two scaffolding protein that play crucial roles in repair (P. Ohouo, Bastos de Oliveira, Almeida, & Smolka, 2010).

Rtt107 is a multi BRCT domain containing protein, which is part of an extensive protein interaction network (P. Ohouo et al., 2010; Rouse, 2004). Rtt107 binds to a ubiquitin-ligase complex that consists of the Rtt101 cullin, the Mms1 E3-ligase and Mms22. This complex is involved in DNA lesion bypass and is suggested to regulate homologous recombination mediated fork repair (Duro, Vaisica, Brown, & Rouse, 2008). We have recently shown that Rtt107 also binds to the Smc5-Smc6 sumo ligase complex (P. Ohouo et al., 2010). This complex is central to the structural maintenance of chromosomes and plays important roles in repair (X Zhao & Blobel, 2005). Rtt107 also binds to γH2A and thus is believed to promote repair by helping recruit repair factors to sites of damage (Li et al., 2012). Our close analysis of Rtt107's protein interaction network showed that it forms mutually exclusive complexes with the repair factors mentioned above (Unpublished). This finding suggests the involvement of Rtt107 in several repair pathways. In fact, cells lacking members of the different Rtt107-based complexes show different sensitivity depending on the genotoxic conditions (Roberts, Zaidi, Vaisica, Peter, & Brown, 2008).

Slx4 is a highly conserved scaffold for the fork structures specific endonucleases, which in the budding yeast *S. cerevisiae*, associates with the Slx1 (human SLX1) and Rad1/Rad10 (human endonucleases XPF/ERCC1) (Coulon et al.,

2004; Fricke & Brill, 2003). In humans, the scaffolding function of Slx4 extends to the Mus81 endonuclease (Fekairi et al., 2009). While Slx4 has no known catalytic activity, results from *in vitro* analyses suggest that it potentiates the activities of endonucleases it is associated with (Fekairi et al., 2009; Muñoz et al., 2009). Slx4 appears to play critical roles in DNA repair. In the budding yeast for instance, Slx4 is one of six *S. cerevisiae* genes required for viability in the absence of the Sgs1 RecQ helicase (Mullen, Kaliraman, Ibrahim, & Brill, 2001). The involvement of Sgs1 in the processing of stalled forks and the maintenance of genomic instability suggests that Slx4 may play roles in equally important processes. In humans, Slx4 is suggested to coordinate the action of the associated fork structure specific endonucleases in order to promote repair (Muñoz et al., 2009). Furthermore, mammalian Slx4 was recently characterized as a holliday junction resolvase, where it was shown to play important roles in the repair of crossed linked DNA at stalled forks (Fekairi et al., 2009; Svendsen et al., 2009). In fact, the hypersensitivity of SLX4 deficient cells to DNA interstrand crosslinks, a defining feature in Fanconi anemia (Auerbach & Wolman, 1976), has led to the recent characterization of Slx4 as a member of the Fanconi anemia pathway (Garner & Smogorzewska, 2011; Y. Kim et al., 2011). The fact that Fanconi anemia is a rare genetic disorder characterized by increased genomic instability and cancer predisposition further emphasizes the importance of Slx4 (Auerbach, 2009).

Rtt107 forms a stable complex with Slx4 and amongst the different Rtt107-based complexes, Mec1 is suggested to play a direct role in the repair function of the Rtt107-Slx4 complex (Flott & Rouse, 2005). This is due to the extensive

phosphorylation of Rtt107 and Slx4 by Mec1 upon replication stress induced by the MMS (Flott & Rouse, 2005). A study from the Rouse group suggested that the Mec1-dependent phosphorylation of Slx4 promotes its nuclease dependent function and enables Slx4 to mediate single-strand annealing, which is a pathway for the repair of DSBs via HR (Flott et al., 2007). Interestingly, in human Slx4 is also a target of ATR (Matsuoka et al., 2007; Mu et al., 2007). However functional role of this phosphorylation remains to be determined.

Our analysis of the Mec1-dependent phosphorylation of Slx4 revealed that it mediates a vital interaction between the Rtt107-Slx4 complex and the checkpoint activator protein Dpb11 (P. Ohouo et al., 2010). Cells lacking Rtt107 or Slx4 display excessive Rad53 activation and sickness upon replication stress brought on by DNA alkylation (P. Y. Ohouo et al., 2013; Roberts et al., 2008; Rouse, 2004). Our work on the functional roles of this interaction showed that Rtt107 and Slx4 down regulate Rad53 activation by binding Dpb11 and γH2A thus restricting Rad9's access to these positive regulators of the checkpoint. Our findings indicate the direct involvement of repair proteins in a previously uncharacterized mode of checkpoint down regulation. I speculate that similar to the shelterin complex at the telomeres, Rtt107 and Slx4 help prevent aberrant checkpoint activation by binding gaps of ssDNA at damaged forks(de Lange, 2005).

REFERENCES

Alabert, C., Bianco, J. N., & Pasero, P. (2009). Differential regulation of homologous recombination at DNA breaks and replication forks by the Mrc1 branch of the S-phase checkpoint. *EMBO J, 28*(8), 1131-1141. doi: 10.1038/emboj.2009.75

Alcasabas, A., Osborn, A., Bachant, J., Hu, F., Werler, P., Bousset, K., . . . Elledge, S. (2001). Mrc1 transduces signals of DNA replication stress to activate Rad53. *Nat Cell Biol, 3*(11), 958-965.

Allen, J. B., Zhou, Z., Siede, W., Friedberg, E. C., & Elledge, S. J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. *Genes Dev, 8*(20), 2401-2415.

Auerbach, A. D. (2009). Fanconi anemia and its diagnosis. *Mutat Res, 668*(1-2), 4-10. doi: 10.1016/j.mrfmmm.2009.01.013

Auerbach, A. D., & Wolman, S. R. (1976). Susceptibility of Fanconi's anaemia fibroblasts to chromosome damage by carcinogens. *Nature, 261*(5560), 494-496.

Azvolinsky, A., Giresi, P. G., Lieb, J. D., & Zakian, V. A. (2009). Highly transcribed RNA polymerase II genes are impediments to replication fork progression in Saccharomyces cerevisiae. *Mol Cell, 34*(6), 722-734. doi: 10.1016/j.molcel.2009.05.022

Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., . . . Vogelstein, B. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science, 244*(4901), 217-221.

Baker, S. J., Markowitz, S., Fearon, E. R., Willson, J. K., & Vogelstein, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science, 249*(4971), 912-915.

Baroni, E., Viscardi, V., Cartagena-Lirola, H., Lucchini, G., & Longhese, M. P. (2004). The functions of budding yeast Sae2 in the DNA damage response require Mec1- and Tel1-dependent phosphorylation. *Mol Cell Biol, 24*(10), 4151-4165.

Bartek, J., Bartkova, J., & Lukas, J. (2007). DNA damage signalling guards against activated oncogenes and tumour progression. *Oncogene, 26*(56), 7773-7779. doi: 10.1038/sj.onc.1210881

Bartkova, J., Horejsí, Z., Koed, K., Krämer, A., Tort, F., Zieger, K., . . . Bartek, J. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature, 434*(7035), 864-870. doi: 10.1038/nature03482

Bassing, C. H., Chua, K. F., Sekiguchi, J., Suh, H., Whitlow, S. R., Fleming, J. C., . . . Alt, F. W. (2002). Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX. *Proc Natl Acad Sci U S A, 99*(12), 8173-8178. doi: 10.1073/pnas.122228699

Bazzi, M., Mantiero, D., Trovesi, C., Lucchini, G., & Longhese, M. P. (2010). Dephosphorylation of gamma H2A by Glc7/protein phosphatase 1 promotes recovery from inhibition of DNA replication. *Mol Cell Biol, 30*(1), 131-145. doi: 10.1128/MCB.01000-09

Beckman, R. A., & Loeb, L. A. (2006). Efficiency of carcinogenesis with and without a mutator mutation. *Proc Natl Acad Sci U S A, 103*(38), 14140-14145. doi: 10.1073/pnas.0606271103

Brown, E. J., & Baltimore, D. (2003). Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. *Genes Dev, 17*(5), 615-628. doi: 10.1101/gad.1067403

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., . . . Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science, 282*(5393), 1497-1501.

Celeste, A., Petersen, S., Romanienko, P. J., Fernandez-Capetillo, O., Chen, H. T., Sedelnikova, O. A., . . . Nussenzweig, A. (2002). Genomic instability in mice lacking histone H2AX. *Science, 296*(5569), 922-927. doi: 10.1126/science.1069398

Chabes, A., Georgieva, B., Domkin, V., Zhao, X., Rothstein, R., & Thelander, L. (2003). Survival of DNA damage in yeast directly depends on increased dNTP levels allowed by relaxed feedback inhibition of ribonucleotide reductase. *Cell, 112*(3), 391-401.

Chen, M. S., Ryan, C. E., & Piwnica-Worms, H. (2003). Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding. *Mol Cell Biol, 23*(21), 7488-7497.

Chen, S., & Zhou, H. (2009). Reconstitution of Rad53 Activation by Mec1 through Adaptor Protein Mrc1. *J Biol Chem, 284*(28), 18593-18604.

Cobb, J., Bjergbaek, L., Shimada, K., Frei, C., & Gasser, S. (2003). DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1. *EMBO J, 22*(16), 4325-4336. doi: 10.1093/emboj/cdg391

Cobb, J. A., Schleker, T., Rojas, V., Bjergbaek, L., Tercero, J. A., & Gasser, S. M. (2005). Replisome instability, fork collapse, and gross chromosomal rearrangements arise synergistically from Mec1 kinase and RecQ helicase mutations. *Genes Dev, 19*(24), 3055-3069. doi: 19/24/3055 [pii]

10.1101/gad.361805

Cortez, D., Guntuku, S., Qin, J., & Elledge, S. (2001). ATR and ATRIP: partners in checkpoint signaling. S*cience, 294(*5547), 1713-1716. doi: 294/5547/1713 [pii]

10.1126/science.1065521

Costanzo, V., Shechter, D., Lupardus, P. J., Cimprich, K. A., Gottesman, M., & Gautier, J. (2003). An ATR- and Cdc7-dependent DNA damage checkpoint that inhibits initiation of DNA replication. Mo*l Cell, 11(1*), 203-213.

Cotta-Ramusino, C., Fachinetti, D., Lucca, C., Doksani, Y., Lopes, M., Sogo, J., & Foiani, M. (2005). Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. Mo*l Cell, 17(1*), 153-159. doi: S109727650400718X [pii]

10.1016/j.molcel.2004.11.032

Coulon, S., Gaillard, P., Chahwan, C., McDonald, W., Yates, J. r., & Russell, P. (2004). Slx1-Slx4 are subunits of a structure-specific endonuclease that maintains ribosomal DNA in fission yeast. Mol *Biol Cell, 15(1)*, 71-80.

Craven, R. J., Greenwell, P. W., Dominska, M., & Petes, T. D. (2002). Regulation of genome stability by TEL1 and MEC1, yeast homologs of the mammalian ATM and ATR genes. Gen*etics, 161(2)*, 493-507.

Cremona, C. A., Sarangi, P., Yang, Y., Hang, L. E., Rahman, S., & Zhao, X. (2012). Extensive DNA damage-induced sumoylation contributes to replication and repair and acts in addition to the mec1 checkpoint. Mol *Cell, 45(3)*, 422-432. doi: 10.1016/j.molcel.2011.11.028

Dai, Y., & Grant, S. (2010). New insights into checkpoint kinase 1 in the DNA damage response signaling network. Cli*n Cancer Res, 16(2)*, 376-383. doi: 10.1158/1078-0432.CCR-09-1029

de la Torre-Ruiz, M., & Lowndes, N. F. (2000). The Saccharomyces cerevisiae DNA damage checkpoint is required for efficient repair of double strand breaks by non-homologous end joining. FEB*S Lett, 467(2*-3), 311-315.

de Lange, T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. Gen*es Dev, 19(18*), 2100-2110. doi: 10.1101/gad.1346005

De Piccoli, G., Katou, Y., Itoh, T., Nakato, R., Shirahige, K., & Labib, K. (2012). Replisome stability at defective DNA replication forks is independent of S phase checkpoint kinases. Mol *Cell, 45(5)*, 696-704. doi: 10.1016/j.molcel.2012.01.007

Desany, B., Alcasabas, A., Bachant, J., & Elledge, S. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Gen*es Dev, 12(18*), 2956-2970.

Desany, B. A., Alcasabas, A. A., Bachant, J. B., & Elledge, S. J. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Gen*es Dev, 12(18*), 2956-2970.

Duro, E., Vaisica, J., Brown, G., & Rouse, J. (2008). Budding yeast Mms22 and Mms1 regulate homologous recombination induced by replisome blockage. DNA *Repair (Amst), 7(5)*, 811-818. doi: S1568-7864(08)00011-6 [pii] 10.1016/j.dnarep.2008.01.007

Ekholm-Reed, S., Spruck, C. H., Sangfelt, O., van Drogen, F., Mueller-Holzner, E., Widschwendter, M., . . . Reed, S. E. (2004). Mutation of hCDC4 leads to cell cycle deregulation of cyclin E in cancer. Canc*er Res, 64(3),* 795-800.

Ellison, V., & Stillman, B. (2003). Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5' recessed DNA. PLoS *Biol, 1(2),* E33. doi: 10.1371/journal.pbio.0000033

Fekairi, S., Scaglione, S., Chahwan, C., Taylor, E., Tissier, A., Coulon, S., . . . Gaillard, P. (2009). Human SLX4 is a Holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases. Cell*, 138(1),* 78-89.

Flott, S., Alabert, C., Toh, G., Toth, R., Sugawara, N., Campbell, D., . . . Rouse, J. (2007). Phosphorylation of Slx4 by Mec1 and Tel1 regulates the single-strand annealing mode of DNA repair in budding yeast. Mol *Cell Biol, 27(18)*, 6433-6445.

Flott, S., & Rouse, J. (2005). Slx4 becomes phosphorylated after DNA damage in a Mec1/Tel1-dependent manner and is required for repair of DNA alkylation damage. Bioc*hem J, 391(Pt* 2), 325-333.

Foiani, M., Pellicioli, A., Lopes, M., Lucca, C., Ferrari, M., Liberi, G., . . . Plevani1, P. (2000). DNA damage checkpoints and DNA replication controls in Saccharomyces cerevisiae. Muta*t Res, 451(1-2*), 187-196.

Fricke, W., & Brill, S. (2003). Slx1-Slx4 is a second structure-specific endonuclease functionally redundant with Sgs1-Top3. Gene*s Dev, 17(14)*, 1768-1778.

Friedel, A., Pike, B., & Gasser, S. (2009). ATR/Mec1: coordinating fork stability and repair. Curr *Opin Cell Biol, 21(2),* 237-244.

Gambus, A., Jones, R. C., Sanchez-Diaz, A., Kanemaki, M., van Deursen, F., Edmondson, R. D., & Labib, K. (2006). GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. Nat *Cell Biol, 8(4),* 358-366. doi: 10.1038/ncb1382

Garner, E., & Smogorzewska, A. (2011). Ubiquitylation and the Fanconi anemia pathway. FEBS *Lett, 585(18)*, 2853-2860. doi: 10.1016/j.febslet.2011.04.078

Gilbert, C. S., Green, C. M., & Lowndes, N. F. (2001). Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. Mol *Cell, 8(1),* 129-136.

Gorgoulis, V. G., Vassiliou, L. V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., . . . Halazonetis, T. D. (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Natu*re, 434(703*5), 907-913. doi: 10.1038/nature03485

Granata, M., Lazzaro, F., Novarina, D., Panigada, D., Puddu, F., Abreu, C. M., . . . Muzi-Falconi, M. (2010). Dynamics of Rad9 chromatin binding and checkpoint function are mediated by its dimerization and are cell cycle-regulated by CDK1 activity. PLoS *Genet, 6(8).* doi: 10.1371/journal.pgen.1001047

Guillemain, G., Ma, E., Mauger, S., Miron, S., Thai, R., Guérois, R., . . . Marsolier-Kergoat, M. C. (2007). Mechanisms of checkpoint kinase Rad53 inactivation after a

double-strand break in Saccharomyces cerevisiae. Mol *Cell Biol, 27(9),* 3378-3389. doi: 10.1128/MCB.00863-06

Hammet, A., Magill, C., Heierhorst, J., & Jackson, S. (2007). Rad9 BRCT domain interaction with phosphorylated H2AX regulates the G1 checkpoint in budding yeast. EMBO *Rep, 8(9),* 851-857.

Hartwell, L. H., & Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Scie*nce, 246(493*0), 629-634.

Hoeijmakers, J. H. (2009). DNA damage, aging, and cancer. N En*gl J Med, 361(15)*, 1475-1485. doi: 10.1056/NEJMra0804615

Huang, M., Zhou, Z., & Elledge, S. J. (1998). The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell*, 94(5),* 595-605. doi: S0092-8674(00)81601-3 [pii]

Huen, M. S., Grant, R., Manke, I., Minn, K., Yu, X., Yaffe, M. B., & Chen, J. (2007). RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. Cell*, 131(5),* 901-914. doi: 10.1016/j.cell.2007.09.041

Jackson, S. P., & Durocher, D. (2013). Regulation of DNA Damage Responses by Ubiquitin and SUMO. Mol *Cell, 49(5),* 795-807. doi: 10.1016/j.molcel.2013.01.017

Katou, Y., Kanoh, Y., Bando, M., Noguchi, H., Tanaka, H., Ashikari, T., . . . Shirahige, K. (2003). S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. Natu*re, 424(695*2), 1078-1083.

Keogh, M. C., Kim, J. A., Downey, M., Fillingham, J., Chowdhury, D., Harrison, J. C., . . . Krogan, N. J. (2006). A phosphatase complex that dephosphorylates

gammaH2AX regulates DNA damage checkpoint recovery. Natur*e, 439(707*5), 497-501. doi: 10.1038/nature04384

Kim, J. A., Hicks, W. M., Li, J., Tay, S. Y., & Haber, J. E. (2011). Protein phosphatases pph3, ptc2, and ptc3 play redundant roles in DNA double-strand break repair by homologous recombination. Mol *Cell Biol, 31(3),* 507-516. doi: 10.1128/MCB.01168-10

Kim, Y., Lach, F. P., Desetty, R., Hanenberg, H., Auerbach, A. D., & Smogorzewska, A. (2011). Mutations of the SLX4 gene in Fanconi anemia. Nat *Genet, 43(2),* 142-146. doi: 10.1038/ng.750

Kondo, T., Matsumoto, K., & Sugimoto, K. (1999). Role of a complex containing Rad17, Mec3, and Ddc1 in the yeast DNA damage checkpoint pathway. Mol *Cell Biol, 19(2),* 1136-1143.

Kumar, S., & Burgers, P. M. (2013). Lagging strand maturation factor Dna2 is a component of the replication checkpoint initiation machinery. Gene*s Dev, 27(3),* 313-321. doi: 10.1101/gad.204750.112

Labib, K., & De Piccoli, G. (2011). Surviving chromosome replication: the many roles of the S-phase checkpoint pathway. Phil*os Trans R Soc Lond B Biol Sci, 366(1584*), 3554-3561. doi: 10.1098/rstb.2011.0071

Lee, S., Schwartz, M., Duong, J., & Stern, D. (2003). Rad53 phosphorylation site clusters are important for Rad53 regulation and signaling. Mol *Cell Biol, 23(17)*, 6300-6314.

Lengauer, C., Kinzler, K. W., & Vogelstein, B. (1998). Genetic instabilities in human cancers. Natur*e, 396(6712*), 643-649. doi: 10.1038/25292

Lengronne, A., & Schwob, E. (2002). The yeast CDK inhibitor Sic1 prevents genomic instability by promoting replication origin licensing in late G(1). Mol *Cell, 9(5),* 1067-1078.

Leroy, C., Lee, S. E., Vaze, M. B., Ochsenbein, F., Ochsenbien, F., Guerois, R., . . . Marsolier-Kergoat, M. C. (2003). PP2C phosphatases Ptc2 and Ptc3 are required for DNA checkpoint inactivation after a double-strand break. Mol *Cell, 11(3),* 827-835.

Li, X., Liu, K., Li, F., Wang, J., Huang, H., Wu, J., & Shi, Y. (2012). Structure of C-terminal tandem BRCT repeats of Rtt107 protein reveals critical role in interaction with phosphorylated histone H2A during DNA damage repair. J Bi*ol Chem, 287(12)*, 9137-9146. doi: 10.1074/jbc.M111.311860

Liang, F., & Wang, Y. (2007). DNA damage checkpoints inhibit mitotic exit by two different mechanisms. Mol *Cell Biol, 27(14)*, 5067-5078. doi: 10.1128/MCB.00095-07

Liao, H., Yuan, C., Su, M., Yongkiettrakul, S., Qin, D., Li, H., . . . Tsai, M. (2000). Structure of the FHA1 domain of yeast Rad53 and identification of binding sites for both FHA1 and its target protein Rad9. J Mo*l Biol, 304(5),* 941-951.

Lopes, M., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., . . . Foiani, M. (2001). The DNA replication checkpoint response stabilizes stalled replication forks. Natu*re, 412(6846*), 557-561. doi: 35087613 [pii]

10.1038/35087613

Lopez-Mosqueda, J., Maas, N. L., Jonsson, Z. O., Defazio-Eli, L. G., Wohlschlegel, J., & Toczyski, D. P. (2010). Damage-induced phosphorylation of Sld3 is important

to block late origin firing. Nature, *467(7314*), 479-483. doi: 10.1038/nature09377

Lowe, S. W., Cepero, E., & Evan, G. (2004). Intrinsic tumour suppression. Nature, *432(7015*), 307-315. doi: 10.1038/nature03098

Lucca, C., Vanoli, F., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Haber, J., & Foiani, M. (2004). Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing. Oncogene, *23(6),* 1206-1213. doi: 10.1038/sj.onc.1207199

Luciani, M. G., Oehlmann, M., & Blow, J. J. (2004). Characterization of a novel ATR-dependent, Chk1-independent, intra-S-phase checkpoint that suppresses initiation of replication in Xenopus. J Cell *Sci, 117(Pt 2*5), 6019-6030. doi: 10.1242/jcs.01400

Majka, J., Binz, S. K., Wold, M. S., & Burgers, P. M. (2006). Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions. J Biol *Chem, 281(38),* 27855-27861. doi: 10.1074/jbc.M605176200

Marchetti, M. A., Kumar, S., Hartsuiker, E., Maftahi, M., Carr, A. M., Freyer, G. A., . . . Huberman, J. A. (2002). A single unbranched S-phase DNA damage and replication fork blockage checkpoint pathway. Proc *Natl Acad Sci U S A, 99(11),* 7472-7477. doi: 10.1073/pnas.112702399

Masumoto, H., Muramatsu, S., Kamimura, Y., & Araki, H. (2002). S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. Nature, *415(6872*), 651-655. doi: nature713 [pii]

10.1038/nature713

Masumoto, H., Sugino, A., & Araki, H. (2000). Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast. Mol Ce*ll Biol, 20(8), 2*809-2817.

Matsuoka, S., Ballif, B., Smogorzewska, A., McDonald, E. r., Hurov, K., Luo, J., . . . Elledge, S. (2007). ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Scienc*e, 316(5828)*, 1160-1166. doi: 316/5828/1160 [pii]

10.1126/science.1140321

Morin, I., Ngo, H. P., Greenall, A., Zubko, M. K., Morrice, N., & Lydall, D. (2008). Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response. EMBO J, *27(18), 2*400-2410. doi: 10.1038/emboj.2008.171

Mu, J. J., Wang, Y., Luo, H., Leng, M., Zhang, J., Yang, T., . . . Qin, J. (2007). A proteomic analysis of ataxia telangiectasia-mutated (ATM)/ATM-Rad3-related (ATR) substrates identifies the ubiquitin-proteasome system as a regulator for DNA damage checkpoints. J Biol *Chem, 282(24), 1*7330-17334. doi: 10.1074/jbc.C700079200

Mullen, J., Kaliraman, V., Ibrahim, S., & Brill, S. (2001). Requirement for three novel protein complexes in the absence of the Sgs1 DNA helicase in Saccharomyces cerevisiae. Genetic*s, 157(1), 10*3-118.

Muñoz, I., Hain, K., Déclais, A., Gardiner, M., Toh, G., Sanchez-Pulido, L., . . . Rouse, J. (2009). Coordination of structure-specific nucleases by human SLX4/BTBD12 is required for DNA repair. Mol Cel*l, 35(1), 11*6-127.

Myung, K., Datta, A., & Kolodner, R. D. (2001). Suppression of spontaneous chromosomal rearrangements by S phase checkpoint functions in Saccharomyces cerevisiae. Cell, 1*04(3), 39*7-408.

Myung, K., & Kolodner, R. (2002). Suppression of genome instability by redundant S-phase checkpoint pathways in Saccharomyces cerevisiae. Proc Na*tl Acad Sci U S A, 99(7), 45*00-4507. doi: 062702199 [pii]

10.1073/pnas.062702199

Navadgi-Patil, V., & Burgers, P. (2008). Yeast DNA replication protein Dpb11 activates the Mec1/ATR checkpoint kinase. J Biol C*hem, 283(51), 35*853-35859. doi: M807435200 [pii]

10.1074/jbc.M807435200

Navadgi-Patil, V. M., & Burgers, P. M. (2009). The unstructured C-terminal tail of the 9-1-1 clamp subunit Ddc1 activates Mec1/ATR via two distinct mechanisms. Mol Cell, *36(5), 743*-753. doi: 10.1016/j.molcel.2009.10.014

Naylor, M., Li, J., Osborn, A., & Elledge, S. (2009). Mrc1 phosphorylation in response to DNA replication stress is required for Mec1 accumulation at the stalled fork. Proc Natl *Acad Sci U S A.*

*O'Neil*l, B. M., Szyjka, S. J., Lis, E. T., Bailey, A. O., Yates, J. R., Aparicio, O. M., & Romesberg, F. E. (2007). Pph3-Psy2 is a phosphatase complex required for Rad53 dephosphorylation and replication fork restart during recovery from DNA damage. Proc Natl *Acad Sci U S A, 104(22), 929*0-9295. doi: 10.1073/pnas.0703252104

Ohouo, P., Bastos de Oliveira, F., Almeida, B., & Smolka, M. (2010). DNA damage signaling recruits the Rtt107-Slx4 scaffolds via Dpb11 to mediate replication stress response. Mol Cell, *39(2), 300*-306. doi: S1097-2765(10)00460-0 [pii]
10.1016/j.molcel.2010.06.019

Ohouo, P. Y., Bastos de Oliveira, F. M., Liu, Y., Ma, C. J., & Smolka, M. B. (2013). DNA-repair scaffolds dampen checkpoint signalling by counteracting the adaptor Rad9. Nature, 49*3(7430), 12*0-124. doi: 10.1038/nature11658

Pfander, B., & Diffley, J. F. (2011). Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment. EMBO J, 30*(24), 4897*-4907. doi: emboj2011345 [pii]
10.1038/emboj.2011.345

Phillips, D. H., Hewer, A., Martin, C. N., Garner, R. C., & King, M. M. (1988). Correlation of DNA adduct levels in human lung with cigarette smoking. Nature, 336*(6201), 790*-792. doi: 10.1038/336790a02909886

Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., & Vogelstein, B. (1997). A model for p53-induced apoptosis. Nature, 389*(6648), 300*-305. doi: 10.1038/38525

Raveendranathan, M., Chattopadhyay, S., Bolon, Y. T., Haworth, J., Clarke, D. J., & Bielinsky, A. K. (2006). Genome-wide replication profiles of S-phase checkpoint mutants reveal fragile sites in yeast. EMBO J, 25(*15), 3627*-3639. doi: 10.1038/sj.emboj.7601251

Roberts, T., Zaidi, I., Vaisica, J., Peter, M., & Brown, G. (2008). Regulation of rtt107 recruitment to stalled DNA replication forks by the cullin rtt101 and the rtt109 acetyltransferase. Mol Biol Ce*ll, 19(1), 171-18*0.

Rouse, J. (2004). Esc4p, a new target of Mec1p (ATR), promotes resumption of DNA synthesis after DNA damage. EMBO J, 23(*5), 1188-1*197.

Rouse, J., & Jackson, S. (2000). LCD1: an essential gene involved in checkpoint control and regulation of the MEC1 signalling pathway in Saccharomyces cerevisiae. EMBO J, 19(*21), 5801*-5812. doi: 10.1093/emboj/19.21.5801

Sancar, A., Lindsey-Boltz, L. A., Unsal-Kaçmaz, K., & Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Bi*ochem, 73, 39-85. do*i: 10.1146/annurev.biochem.73.011303.073723

Santocanale, C., & Diffley, J. (1998). A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature, 395*(6702), 615*-618.

Schwartz, M., Lee, S., Duong, J., Eminaga, S., & Stern, D. FHA domain-mediated DNA checkpoint regulation of Rad53. Cell Cycle, *2(4), 384-396*.

Schwartz, M. F., Duong, J. K., Sun, Z., Morrow, J. S., Pradhan, D., & Stern, D. F. (2002). Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint. Mol Cell, 9*(5), 1055-1*065.

Segurado, M., & Diffley, J. F. (2008). Separate roles for the DNA damage checkpoint protein kinases in stabilizing DNA replication forks. Genes Dev, *22(13), 1816*-1827. doi: 22/13/1816 [pii]

10.1101/gad.477208

Smolka, M., Albuquerque, C., Chen, S., & Zhou, H. (2007). Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. Proc Natl Ac*ad Sci U S A, 104(25), 10364*-10369.

Smolka, M., Chen, S., Maddox, P., Enserink, J., Albuquerque, C., Wei, X., . . . Zhou, H. (2006). An FHA domain-mediated protein interaction network of Rad53 reveals its role in polarized cell growth. J Cell Biol, *175(5), 743-753*.

Sogo, J. M., Lopes, M., & Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science, 297*(5581), 599*-602. doi: 10.1126/science.1074023

Spruck, C. H., Won, K. A., & Reed, S. I. (1999). Deregulated cyclin E induces chromosome instability. Nature, 401(*6750), 297*-300. doi: 10.1038/45836

Sun, Z., Hsiao, J., Fay, D. S., & Stern, D. F. (1998). Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Science, 281*(5374), 272*-274.

Svendsen, J., Smogorzewska, A., Sowa, M., O'Connell, B., Gygi, S., Elledge, S., & Harper, J. (2009). Mammalian BTBD12/SLX4 assembles a Holliday junction resolvase and is required for DNA repair. Cell, 138(1)*, 63-77.*

Szyjka, S., Aparicio, J., Viggiani, C., Knott, S., Xu, W., Tavaré, S., & Aparicio, O. (2008). Rad53 regulates replication fork restart after DNA damage in Saccharomyces cerevisiae. Genes Dev, 2*2(14), 1906-1*920.

Tak, Y. S., Tanaka, Y., Endo, S., Kamimura, Y., & Araki, H. (2006). A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2-Dpb11. EMBO J, 25(9*), 1987-19*96. doi: 10.1038/sj.emboj.7601075

Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A., & Araki, H. (2003). GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. Genes Dev, 1*7(9), 1153-11*65.

Tanaka, S., & Diffley, J. F. (2002). Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. Genes Dev, 1*6(20), 2639-2*649. doi: 10.1101/gad.1011002

Tercero, J., Longhese, M., & Diffley, J. (2003). A central role for DNA replication forks in checkpoint activation and response. Mol Cell, 11*(5), 1323-13*36.

Tercero, J. A., & Diffley, J. F. (2001). Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature, 412(*6846), 553*-557. doi: 10.1038/35087607

Thiriet, C., & Hayes, J. J. (2005). Replication-independent core histone dynamics at transcriptionally active loci in vivo. Genes Dev, 1*9(6), 677-682*. doi: 10.1101/gad.1265205

Tourrière, H., Versini, G., Cordón-Preciado, V., Alabert, C., & Pasero, P. (2005). Mrc1 and Tof1 promote replication fork progression and recovery independently of Rad53. Mol Cell, 19*(5), 699-706*.

Usui, T., Foster, S., & Petrini, J. (2009). Maintenance of the DNA-damage checkpoint requires DNA-damage-induced mediator protein oligomerization. Mol Cell, 33*(2), 147-159*.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T. K., Hampton, G. M., & Wahl, G. M. (2002). c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell, 9(*5), 1031-10*44.

van Attikum, H., & Gasser, S. M. (2009). Crosstalk between histone modifications during the DNA damage response. Trends Cell *Biol, 19(5), 207-217*. doi: 10.1016/j.tcb.2009.03.001

van Vugt, M. A., Brás, A., & Medema, R. H. (2004). Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. Mol Cell, 15*(5), 799-811*. doi: 10.1016/j.molcel.2004.07.015

Vialard, J. E., Gilbert, C. S., Green, C. M., & Lowndes, N. F. (1998). The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. EMBO J, 17(1*9), 5679-5*688. doi: 10.1093/emboj/17.19.5679

Vidanes, G. M., Sweeney, F. D., Galicia, S., Cheung, S., Doyle, J. P., Durocher, D., & Toczyski, D. P. (2010). CDC5 inhibits the hyperphosphorylation of the checkpoint kinase Rad53, leading to checkpoint adaptation. PLoS Biol, 8*(1), e1000286*. doi: 10.1371/journal.pbio.1000286

Wang, G., Tong, X., Weng, S., & Zhou, H. (2012). Multiple phosphorylation of Rad9 by CDK is required for DNA damage checkpoint activation. Cell Cycle, *11(20), 3792-3*800. doi: 10.4161/cc.21987

Wang, X., Andreassen, P. R., & D'Andrea, A. D. (2004). Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. Mol Cell Bio*l, 24(13), 5850-5*862. doi: 10.1128/MCB.24.13.5850-5862.2004

Weinert, T. (1998a). DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. Cell, 94(5), *555-558*.

Weinert, T. (1998b). DNA damage checkpoints update: getting molecular. Curr Opin Ge*net Dev, 8(2), 185-193*.

Weinert, T. A., & Hartwell, L. H. (1988). The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science, 241*(4863), 317*-322.

Wold, M. S. (1997). Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu Rev Bio*chem, 66, 61-92. doi*: 10.1146/annurev.biochem.66.1.61

Wysocki, R., Javaheri, A., Allard, S., Sha, F., Côté, J., & Kron, S. J. (2005). Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. Mol Cell Bio*l, 25(19), 8430-8*443. doi: 25/19/8430 [pii]

10.1128/MCB.25.19.8430-8443.2005

Zegerman, P., & Diffley, J. F. (2010). Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. Nature, 467(7*314), 474-4*78. doi: 10.1038/nature09373

Zhao, X., & Blobel, G. (2005). A SUMO ligase is part of a nuclear multiprotein complex that affects DNA repair and chromosomal organization. Proc Natl Aca*d Sci U S A, 102(13), 4777-47*82. doi: 0500537102 [pii]

10.1073/pnas.0500537102

Zhao, X., Muller, E. G., & Rothstein, R. (1998). A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. Mol Cell, 2(3)*, 329-340.*

Zhao, X., & Rothstein, R. (2002). The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1. Proc Natl Acad *Sci U S A, 99(6), 3746-3751*. doi: 10.1073/pnas.062502299

Zheng, P., Fay, D. S., Burton, J., Xiao, H., Pinkham, J. L., & Stern, D. F. (1993). SPK1 is an essential S-phase-specific gene of Saccharomyces cerevisiae that encodes a nuclear serine/threonine/tyrosine kinase. Mol Cell Biol, *13(9), 5829-5842*.

Zhu, Z., Chung, W. H., Shim, E. Y., Lee, S. E., & Ira, G. (2008). Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell, 134(6), *981-994.* doi: 10.1016/j.cell.2008.08.037

Zou, L., & Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science, 300(5*625), 1542-1*548. doi: 10.1126/science.1083430

CHAPTER 2[1]

DNA Damage Signaling Recruits the Rtt107-Slx4 Scaffolds via Dpb11 to Mediate Replication Stress Response.

INTRODUCTION

The ability of cells to maintain the integrity of replication forks is essential to prevent genomic instability and requires the action of the central DNA damage checkpoint kinase ATR^{Mec1}. In *Saccharomyces cerevisiae*, cells lacking Mec1 accumulate spontaneous gross chromosomal rearrangements and exhibit hypersensitivity to agents that perturb DNA replication (Myung et al., 2001 and Weinert et al., 1994). In response to replication stress, Mec1 is recruited to replication forks and is subsequently activated by the replication factor Dpb11 (human TopBP1) (Mordes et al., 2008 and Navadgi-Patil and Burgers, 2008). Mec1 then phosphorylates a number of targets in the DNA replication and repair machineries to promote fork stabilization, recovery of damaged forks, and replication through damaged DNA (Cobb et al., 2003, Lopes et al., 2001 and Tercero and Diffley, 2001). Though the mechanisms of Mec1-dependent stabilization of replication forks are being elucidated, less is known about the mechanisms of how Mec1 regulates fork repair.

Rtt107 and Slx4 are two scaffold proteins involved in DNA repair that are extensively phosphorylated by Mec1 and are important for the recovery of damaged replication forks (Flott and Rouse, 2005 and Rouse, 2004). Both proteins stably interact with each other and are required for resistance to the

[1] Some of the experiments presented in this chapter were published in Ohouo et al., 2010.

DNA alkylating agent methylmethane sulfonate (MMS) (Roberts et al., 2006 and Rouse, 2004). This requirement is likely related to the role of Rtt107 and Slx4 in mediating fork restart and resumption of DNA synthesis after MMS-induced fork blockage. Rtt107 appears to also have Slx4-independent functions, as *rtt107Δ* cells are sensitive to a wide spectrum of replication stress-inducing agents, whereas *slx4Δ* cells are specifically sensitive to MMS (Roberts et al., 2008). As bona fide scaffolds, Rtt107 and Slx4 interact with numerous DNA repair factors. Rtt107 is a multi-BRCT domain containing protein that was previously shown to interact with the Rtt101-Mms1-Mms22 complex, a ubiquitin-ligase that is required for accurate replication through damaged templates (Luke et al., 2006 and Roberts et al., 2008). Slx4 has been reported to bind Slx1 to form a structure-specific endonuclease important for maintaining the stability of the rDNA locus in yeast (Coulon et al., 2004 and Fricke and Brill, 2003). Recently, the human ortholog of Slx4 was identified and shown to form a Holliday junction resolvase together with the human ortholog of Slx1 (Fekairi et al., 2009 and Svendsen et al., 2009). Such resolvase activity was shown to be important to prevent spontaneous and drug-induced genomic instability, probably through the rescue of stalled or collapsed replication forks. In humans, Slx4 was shown to have Slx1-independent functions in genome maintenance by acting as a docking platform for other structure-specific endonucleases such as XPF-ERCC1Rad1-Rad10 and MUS81-EME1Mus81-Mms4. Of interest, human Slx4 is also phosphorylated by ATR^{Mec1}. Despite the importance of Slx4 and Rtt107 in the replication stress response, the molecular mechanisms of how they function are incompletely

understood, and the role of Mec1-mediated phosphorylation of these proteins remains enigmatic.

Here, I hypothesized that Mec1-dependent phosphorylation of Rtt107 and/or Slx4 regulates their scaffold function by promoting physical interactions with other players in the replication stress response. I used a proteomic approach to identify proteins that interact with Rtt107-Slx4 during replication stress in a Mec1-dependent manner. This led me to the finding that Mec1 mediates a key interaction between Rtt107-Slx4 and Dpb11. I present a detailed characterization of the molecular mechanism of the interaction and show that it is important for the response to replication forks blocked by DNA alkylation.

MATERIALS AND METHODS

Plasmids and Yeast Strains

All yeast strains and plasmids used in this study are described in tables 2.1 and 2.2. Standard methodologies were used to generate the plasmids containing c-terminal-tagged RTT107-3XHA and SLX4-3XFLAG. DNA sequences were amplified from genomic DNA and cloned under the control of their native promoters into the plasmid pRS416 (Stratagene) to generate pMBS163 and pMBS213, respectively. SLX4 was also cloned into pFA6a-KanMX6 plasmid, followed by site-directed mutagenesis reactions (to generate SLX4-3MUT and SLX4-7MUT mutants) and integrated into the endogenous SLX4 locus in *slx4Δ* cells. All point mutations in this work were generated using QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). All plasmids were sequenced and are available upon request.

Yeast strains generated in this study are derived from MBS164 (YPH499, congenic to S288C), unless otherwise stated. All tags were integrated by homologous recombination at the c-terminus of the genes original chromosomal loci. Tagged proteins were verified by western blotting. Doubling times of cells harboring the tagged proteins were assessed by growth in MMS to ensure that they behaved similarly to wild type. Sensitivity of SLX4-7MUT to MMS (as shown in Figure 2.4C) was also confirmed in a different background using MBS661 and MBS665 strains derived from a freshly sporulated diploid.

Analysis of Rtt107-Interacting Proteins by SILAC, HILIC and LC-MS/MS

(protocol used for experiments shown in Figures 2.1A and 2.2B) The strains MBS164 (wild-type), MBS266 (Rtt107-HA) or MBS402 (Rtt107-HA in *mec1Δ*) were grown to O.D.600 of 0.4 in 400 mL of -Arg -Lys dropout media ("light" version complemented with normal arginine and lysine; "heavy" version complemented with L-Lysine 13C6, 15N2.HCl and L-Arginine 13C6, 15N4.HCl) and treated with 0.04% MMS for 2 hours. After centrifugation, pellets were kept at -80C prior to cell lysis. Approximately 0.6 g of cell pellet of each strain was lysed by bead beating at 4°C in 4 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, Complete EDTA-free protease inhibitor cocktail (Roche), 5 mM sodium fluoride, 10 mM B-glycerol-phosphate). Lysate was incubated with anti-HA agarose resin (Sigma) for 4 hours at 4°C. After 3 washes with lysis buffer, bound proteins were eluted with 3 resin volumes of elution buffer (100 mM Tris-HCl pH 8.0, 1% SDS). Eluted proteins from normal or heavy media were mixed together, reduced, alkylated and then precipitated. For mass spectrometry experiments, cells were grown in −Arg −Lys dropout media (“light” version complemented with normal arginine and lysine; “heavy” version complemented with lysine 13C6, 15N2 and arginine 13C6, 15N4) and treated with 0.04% MMS for 2 hr. For co-IP experiments, cells were grown in YPD media and treated with 0.04% MMS for 2 hr. Cell lysis and pull-down experiments for either MS analysis or western blotting experiments are described below.

HILIC Fractionation and Mass Spectrometry Analysis

Proteins from pull-down experiments were solubilized with 200uL of 2M urea, 50mM Tris-HCL, pH 8.0, 150mM NaCl, followed by the addition of 2 ug of TPCK treated trypsin. Digestion was performed overnight at 37°C and then trifluoroacetic acid and formic acid were added to a final concentration of 0.2 %. Peptides were de-salted with Sep-Pak C18 column (Waters), dried in a speed-vac, reconstituted in 85uL of a solution containing 80% acetonitrile and 1% formic acid and fractionated by Hidrophilic Interaction Chromatography (HILIC) as previously reported (Albuquerque et al., 2008). HILIC fractions were dried in a speed-vac, reconstituted in 0.1% trifluoroacetic acid and analyzed by LC-MS/MS using 125uM ID capillary C18 column and an Orbitrap XL mass spectrometer coupled with an Eksigent nano-flow system.

Database search and data analysis

Database search was performed using the Sorcerer system (Sagen) running Sequest program. After searching a target-decoy budding yeast database, results were filter based on Probability Score to achieve a 1 % false positive rate. Quantitation of heavy / light peptide isotope ratios was performed using the Xpress program as previously described (Smolka et al., 2007). All results were also manually inspected.

Cell Culture and Pull-Down Procedure for Coimmunoprecipitation Experiments

Cells with the indicated epitope tags were grown to O.D.600 of 0.6 in 100 mL of YPD media and treated with 0.04% MMS for 2 hours. After centrifugation, pellets were kept at -80C prior to cell lysis. Approximately 0.1 g of cell pellet of each strain was lysed by bead beating at 4°C in 1 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, Complete EDTA-free protease inhibitor cocktail (Roche), 5 mM sodium fluoride, 10 mM B-glycerol-phosphate). Lysate was incubated with either anti-HA or anti-Flag agarose resin (Sigma) for 4 hours at 4°C. After 3 washes with lysis buffer, bound proteins were eluted with 3 resin volumes of elution buffer (for anti-HA: 100 mM Tris-HCl pH 8.0, 1% SDS; for anti-Flag: 0.5ug/mL of FLAG peptide in 100mM Tris, 0.2% Tergitol).

Table 2-1 Yeast strains used in this study

Name	Genotype
MBS164	MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1 bar1Δ:HIS3
MBS192	Like MBS164 only RAD53-6xHis-3xHA::KanMX6
MBS266	Like MBS164 only RTT107-6xHis-3xHA::KanMX6
MBS294	Like MBS164 only rtt107Δ::KanMX6
MBS402	Like MBS266 only mec1Δ::URA3
MBS448	Like MBS164 only DPB11-6xHis-3xHA::KanMX6 KanMX6Δ::LEU2
MBS449	Like MBS164 only MMS21-6xHis-3xHA::KanMX6
MBS450	Like MBS164 only RTT107-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2 DPB11-6xHis-3xHA::KanMX6
MBS454	Like MBS164 only MMS21-6xHis-3xHA::KanMX6 KanMX6Δ::LEU2 RTT107-6XHIS-3XFLAG::KanMX6
MBS465	Like MBS164 only RTT107-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2 DPB11-6xHis-3xHA::KanMX6 mec1Δ::URA3
MBS467	Like MBS164 only RTT107-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2 DPB11-6xHis-3xHA::KanMX6 rad53Δ::URA3
MBS469	Like MBS164 only RTT107-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2 DPB11-6xHis-3xHA::KanMX6 slx4Δ::URA3
MBS471	Like MBS164 only DPB11-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2
MBS472	Like MBS164 only RTT107-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2
MBS484	Like MBS164 only SLX1-6xHis-3xHA::KanMX6

Table 2-1 Yeast strains used in this study (continued)

MBS490	Like MBS164 only SMC6-6xHis-3xHA::KanMX6
MBS496	Like MBS164 only RTT107-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2 SMC6-6xHis-3xHA::KanMX6
MBS509	Like MBS164 only DPB11-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2 RTT107-6xHis-3xHA::KanMX6
MBS542	Like MBS164 only DPB11-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2 SLX1-6xHis-3xHA::KanMx6
MBS569	Like MBS164 only DPB11-6XHIS-3XFLAG::KanMX6 KanMX6Δ::LEU2 SLX1-6xHis-3xHA::KanMx7 rtt107Δ::URA3
MBS571	Like MBS164 only DPB11-6xHis-3xHA::KanMX6 KanMX6Δ::LEU2 SLX4-6XHIS-3XFLAG::KanMX6
MBS573	Like MBS164 only DPB11-6xHis-3xHA::KanMX6 KanMX6Δ::LEU2 SLX4-6XHIS-3XFLAG::KanMX6 rtt107Δ::URA3
MBS579	Like MBS164 only DPB11-6xHis-3xHA::KanMX6 KanMX6Δ::LEU2 slx4Δ::KanMX6
MBS621	Like MBS164 only DPB11-6xHis-3xHA::KanMX6 KanMX6Δ::LEU2 SLX4-6XHIS-3XFLAG::KanMX6 slx1Δ::URA
MBS628	Like MBS164 only slx4Δ::URA3 ura3Δ::SLX4-6XHIS-3XFLAG KanMX6
MBS629	Like MBS164 only slx4Δ::URA3 ura3Δ::SLX4_3MUT-6XHIS-3XFLAG KanMX6
MBS630	Like MBS164 only slx4Δ::URA3 ura3Δ::SLX4_7MUT-6XHIS-3XFLAG KanMX6
MBS661	MATa ura3-52, trp1-63, his3-200
MBS665	Like MBS661 only ura3Δ::SLX4_7MUT-6XHIS-3XFLAG KanMX6
MBS738	Like MBS164 only Dpb11-6XHIS-3HA::KanMX6 KanMX6Δ::LEU Orc6-6XHIS-3HA::KanMX6

Table 2-2 Plasmids used to express Slx4 with 3xFLAG in this study

Plasmid name	Plasmid Backbone	Slx4 residues	Mutations
pMBS213	pRS416	1-748 aa	-
pMBS214	pRS416	1-748 aa	3MUT: T457A;T474A;S627A
pMBS215	pRS416	1-748 aa	7MUT: T457A; T474A; S499A; T597A; S627A; S659A; S725A
pMBS218	pFA6a-KanMX6	1-748 aa	-
pMBS219	pFA6a-KanMX6	1-748 aa	3MUT: T457A;T474A;S627A
pMBS220	pFA6a-KanMX6	1-748 aa	7MUT: T457A; T474A; S499A; T597A; S627A;S659A; S725A

RESULTS

Rtt107 Interacts with Numerous Factors Involved in the Repair of Replication Forks

To identify proteins that interact with the Rtt107-Slx4 scaffold unit in the presence of replication stress, our lab devised a proteomic approach based on quantitative mass spectrometry (Figure 2.1A). Rtt107 was used as a bait because epitope-tagged Slx4 was unstable during our pull-down procedure (data not shown). First, cells with untagged Rtt107 and cells containing HA-tagged Rtt107 were treated with 0.04% MMS, a concentration known to efficiently activate DNA damage checkpoint specifically during S phase. After anti-HA pull-down, proteins were identified and quantified by mass spectrometry. Proteins only present in the anti-HA pull-down from tagged cells were considered to be Rtt107-interacting proteins (Figure 2.1B). Using these criteria, ten proteins were found to specifically interact with Rtt107. As shown in Figure 2.1C, all identified binding partners are involved in replication stress response and could be arranged into four distinct protein complexes, as judged by information from Biogrid (http://www.thebiogrid.org). We identified previously unknown interactions with the Mms21 complex, which bears sumo-ligase activity previously reported to be important for repair of damaged forks (Branzei et al., 2006), and with the replication fork protein Dpb11. The binding specificity of these Rtt107-interacting proteins was validated by coimmunoprecipitation (co-IP) experiments and western blotting (Figure 2. 1D). As expected, Rtt107 was found to stably interact with Slx4, as judged by the high number of identified Slx4

peptides (Figure 2.1C). These results reveal an extended landscape of Rtt107-interacting proteins and connect Rtt107 to multiple mechanisms of fork repair.

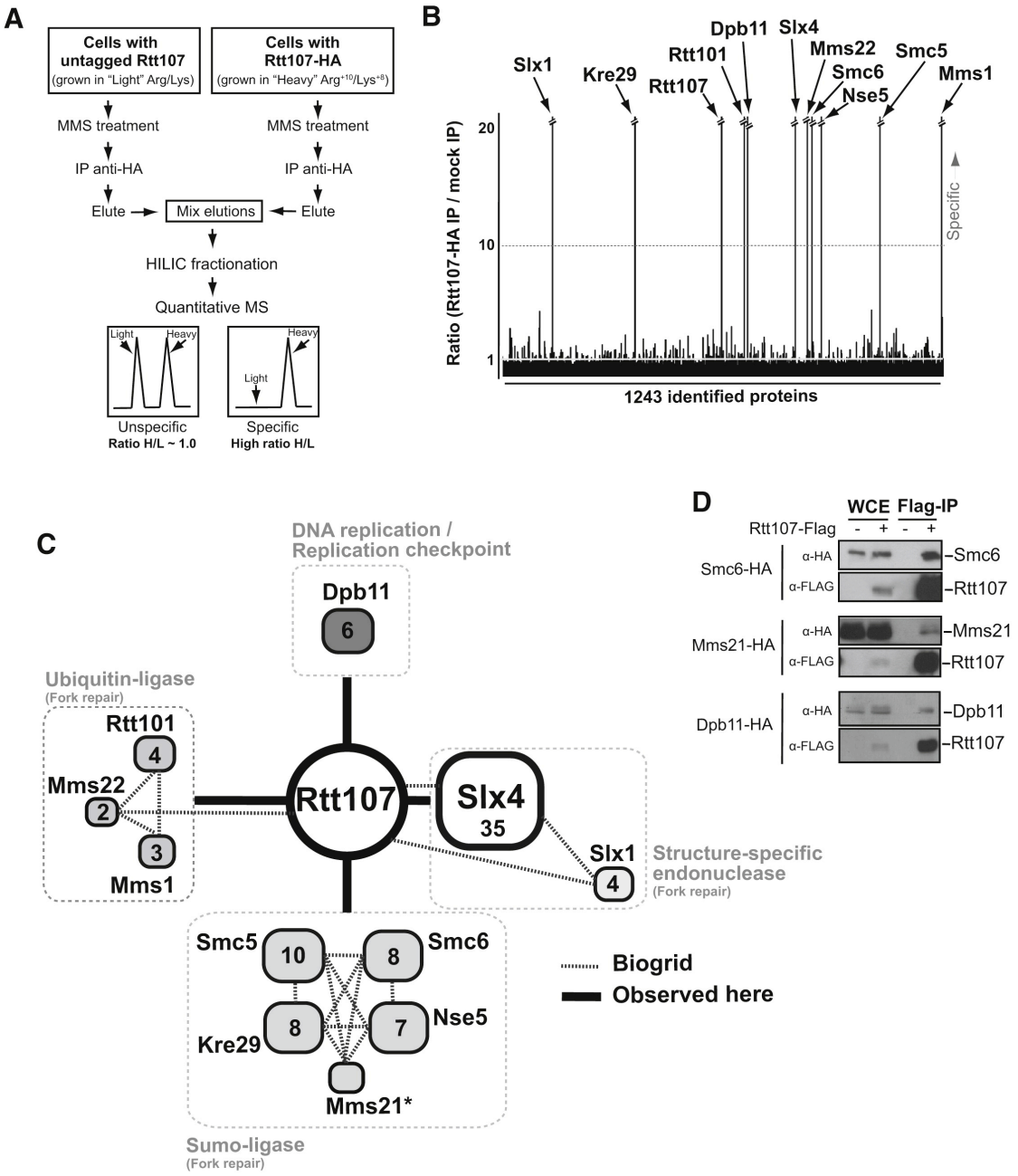

A
Cells with untagged Rtt107 (grown in "Light" Arg/Lys)
Cells with Rtt107-HA (grown in "Heavy" Arg+10/Lys+8)
MMS treatment
MMS treatment
IP anti-HA
IP anti-HA
Elute
Mix elutions
Elute
HILIC fractionation
Quantitative MS
Light
Heavy
Heavy
Light
Unspecific
Ratio H/L ~ 1.0
Specific
High ratio H/L
B
Slx1
Kre29
Rtt101
Rtt107
Dpb11
Slx4
Mms22
Smc6
Nse5
Smc5
Mms1
Ratio (Rtt107-HA IP / mock IP)
20
10
1
Specific
1243 identified proteins
C
DNA replication / Replication checkpoint
Dpb11
6
Ubiquitin-ligase
(Fork repair)
Rtt101
4
Mms22
2
3
Mms1
Rtt107
Slx4
35
Slx1
4
Structure-specific endonuclease
(Fork repair)
Smc5
10
8
Smc6
8
7
Nse5
Kre29
Mms21*
Sumo-ligase
(Fork repair)
Biogrid
Observed here
D
WCE
Flag-IP
Rtt107-Flag
- + - +
Smc6-HA
α-HA
α-FLAG
Smc6
Rtt107
Mms21-HA
α-HA
α-FLAG
Mms21
Rtt107
Dpb11-HA
α-HA
α-FLAG
Dpb11
Rtt107

Figure 2-1

Figure 2.1. Identification of a Network of Rtt107-Interacting Proteins during Replication Stress (A) Outline of the experiment based on quantitative mass spectrometry for the identification of proteins that interact with Rtt107 during replication stress. For details, see text and Supplemental Experimental Procedures. (B) Ratio (Rtt107-HA IP/mock IP) for the 1243 proteins identified and quantified in the analysis outlined in (A). Each ratio corresponds to the geometric mean of the ratios from all peptides identified and quantified for each protein. Proteins with ratios greater than 10 were empirically considered to be specific Rtt107-interacting proteins. (C) Network of Rtt107-interacting proteins in the presence of replication stress. Based on the Biogrid database, the identified interacting proteins were grouped into four distinct complexes, marked with a dotted square. Numbers inside of the squares indicate the number of peptides identified for each protein. ∗Peptides for Mms21 were not detected in the experiment, but the interaction was validated by co-IP experiment. (D) Validation of the identified Rtt107-interacting proteins by co-IP experiments.

The Interaction of Rtt107 with Dpb11 Is Mediated by the DNA Damage Checkpoint

Because Rtt107 and Slx4 are known to be phosphorylated by Mec1 during MMS treatment (Flott and Rouse, 2005, Roberts et al., 2006 and Rouse, 2004), I hypothesized that Mec1-dependent phosphorylation of Rtt107 or Slx4 could regulate their scaffold function by mediating some of the identified protein interactions. To test this, I performed a similar experiment as described in Figure 2.1A, but now comparing anti-HA pull-downs from wild-type and *mec1Δ* cells, both containing HA-tagged Rtt107 and treated with 0.04% MMS (Figure 2. 2A). Figure 2.2B selectively shows the ratios of the specific Rtt107 interactors identified in Figure 2.1B. Although most of the interactions do not change significantly, the interaction between Rtt107 and Dpb11 is dramatically reduced or completely abolished in *mec1Δ* cells. I further validated the result by co-IP and western blotting (Figures 2.2C–E), which revealed that the interaction is also dependent on MMS treatment. Further, deletion of RAD53 caused a significant reduction in the Rtt107-Dpb11 interaction but did not completely abolish it (Figure 2.2E). Taken together, these results show that the interaction between Rtt107 and Dpb11 is dependent on DNA damage checkpoint signaling (Figure 2.2F) and suggest a role for Mec1 in the regulation of fork repair.

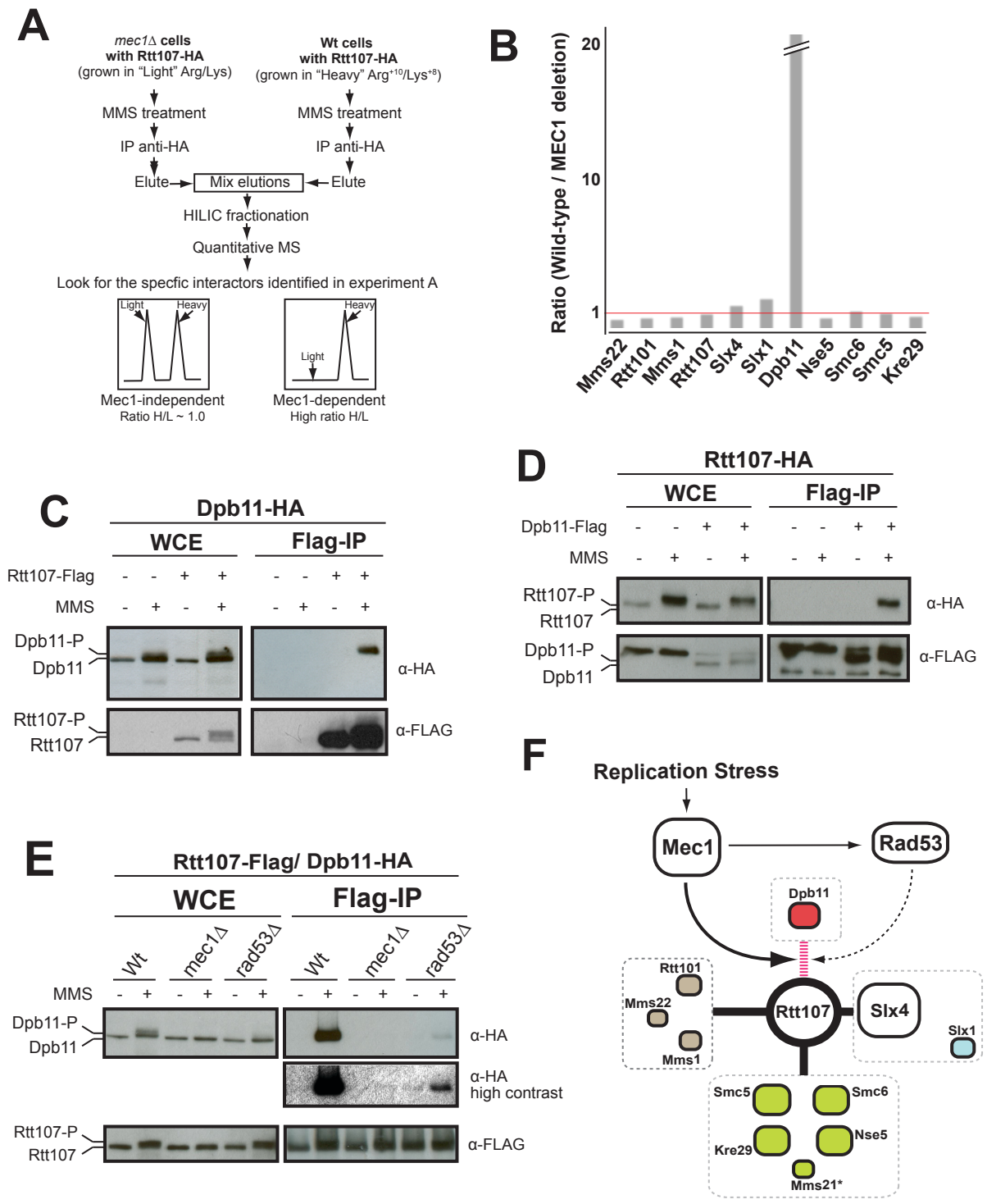

A
mec1Δ cells with Rtt107-HA (grown in "Light" Arg/Lys)
Wt cells with Rtt107-HA (grown in "Heavy" Arg+10/Lys+8)
MMS treatment
IP anti-HA
Elute
Mix elutions
HILIC fractionation
Quantitative MS
Look for the specfic interactors identified in experiment A
Light
Heavy
Mec1-independent
Ratio H/L ~ 1.0
Mec1-dependent
High ratio H/L
B
Ratio (Wild-type / MEC1 deletion)
20
10
1
Mms22
Rtt101
Mms1
Rtt107
Slx4
Slx1
Dpb11
Nse5
Smc6
Smc5
Kre29
C
Dpb11-HA
WCE
Flag-IP
Rtt107-Flag
MMS
Dpb11-P
Dpb11
α-HA
Rtt107-P
Rtt107
α-FLAG
D
Rtt107-HA
WCE
Flag-IP
Dpb11-Flag
MMS
Rtt107-P
Rtt107
α-HA
Dpb11-P
Dpb11
α-FLAG
E
Rtt107-Flag/ Dpb11-HA
WCE
Flag-IP
Wt
mec1Δ
rad53Δ
MMS
Dpb11-P
Dpb11
α-HA
α-HA high contrast
Rtt107-P
Rtt107
α-FLAG
F
Replication Stress
Mec1
Rad53
Dpb11
Rtt101
Mms22
Mms1
Rtt107
Slx4
Slx1
Smc5
Smc6
Nse5
Kre29
Mms21*

Figure 2-2

Figure 2.2. DNA Damage Checkpoint Signaling Is Required for the Interaction between Rtt107 and Dpb11. (A) Outline of the experiment based on quantitative mass spectrometry for determining which of the identified Rtt107 interactions are Mec1 dependent. (B) Graph shows the average ratio (wild-type IP/*mec1Δ* IP) for the specific Rtt107-interacting proteins identified in Figure 2.1B. Ratio for each protein was calculated by averaging the ratios of all corresponding peptides identified and quantified in the analysis. (C–E) Interaction between Dpb11 and Rtt107 is induced by MMS treatment and requires Mec1. WCE, whole-cell extract; ∗, contaminating band. In (E), a high-contrast image is shown to reveal that interaction between Dpb11 and Rtt107 is not completely abolished in *rad53Δ* cells. (F) A model illustrating the role of Mec1 and Rad53 in regulating the interaction between Dpb11 and Rtt107 during replication stress. Dotted arrow indicates that Rad53 is important, but not essential, for the interaction.

Slx4 Mediates the Interaction between Rtt107 and Dpb11

Dpb11 is recruited to stalled or damaged forks by interactions with the 9-1-1 clamp (Ogiwara et al., 2006, Puddu et al., 2008 and Wang and Elledge, 2002) or with the leading-strand polymerase epsilon (Araki et al., 1995 and Masumoto et al., 2000), where it functions to stimulate Mec1 activity. Because Dpb11 is located at forks, it represents a potential anchor for the recruitment of fork repair factors. Thus, the finding that Rtt107 binds Dpb11 could provide a mechanism to explain how the Rtt107 and Slx4 scaffolds are recruited to stalled replication forks. To better understand the mechanism of the interaction, I assessed which of the proteins might be directly binding to Dpb11. Mass spectrometry analysis of a Dpb11 pull-down revealed peptides from Rtt107, Slx4, and Slx1 (data not shown), suggesting that one of these proteins binds Dpb11 directly. I then deleted RTT107 and tested the ability of Dpb11 to interact with Slx4. As shown in Figure 2.3A, deletion of RTT107 strongly reduced the interaction between Dpb11 and Slx4 but did not completely abolish it. This suggests that, although Rtt107 may contribute to stabilizing the Dpb11-Slx4 interaction, it is not absolutely required. Of interest, in the absence of Rtt107, we noticed that the MMS-induced mobility shift of Slx4 is significantly reduced, but not completely abolished (Figure 2.S1A). Next, I found that, whereas Slx1 also coimmunoprecipitates with Dpb11, the deletion of SLX1 had no effect on the ability of Slx4 to bind Dpb11 (Figures 2.S1B and S1C). Finally, I examined the effect of deleting SLX4 on the ability of Rtt107 to bind to Dpb11. Figure 2.3B shows that, in the absence of SLX4, no interaction could be detected between Dpb11 and Rtt107. Collectively; the

results reveal that Slx4 is required for the formation of the Dpb11-Slx4-Rtt107 complex, suggesting that it may directly bind Dpb11. Of interest, Rtt107 plays an important but nonessential role in mediating the interaction.

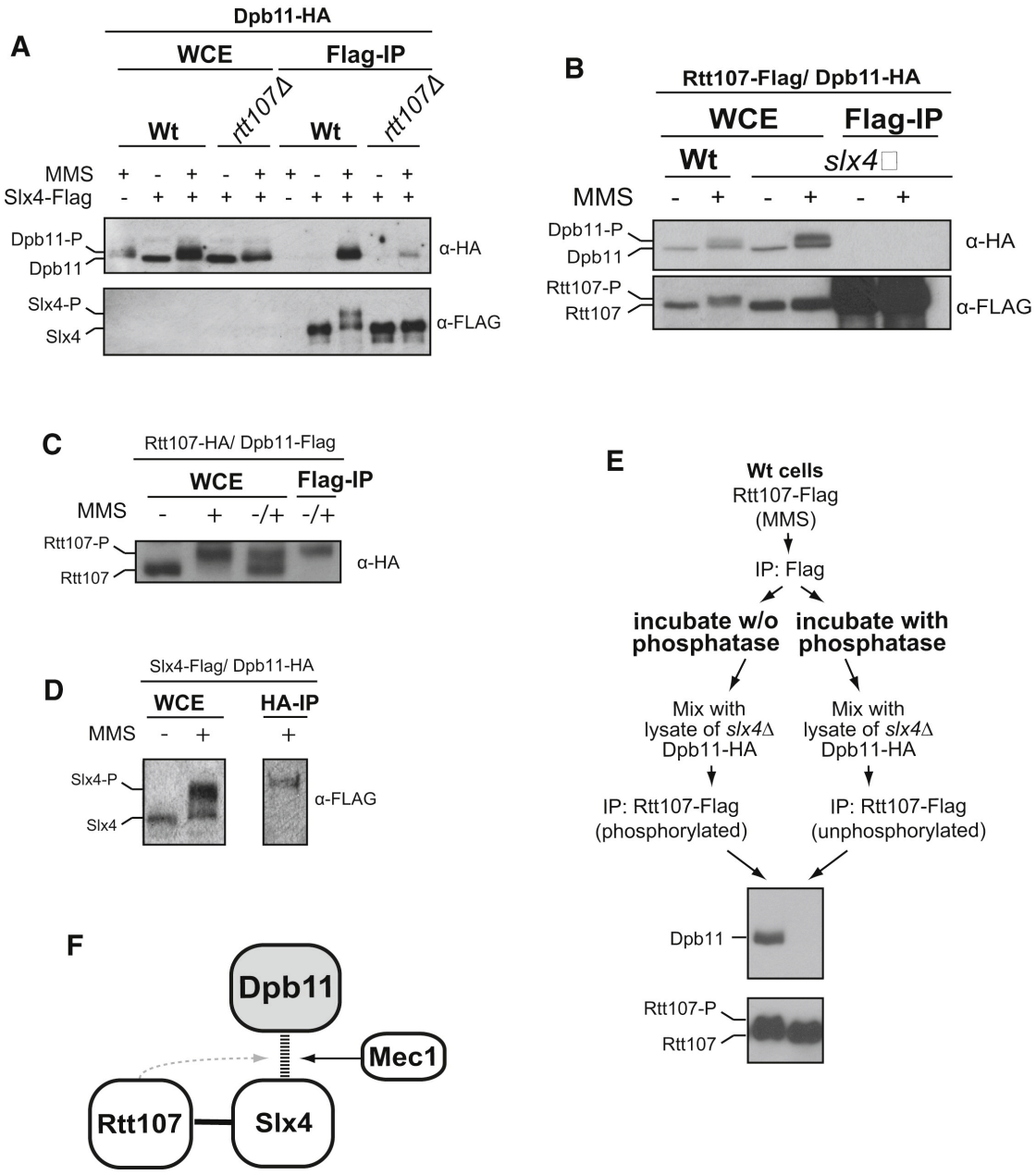

A
Dpb11-HA
WCE
Flag-IP
Wt
rtt107Δ
Wt
rtt107Δ
MMS + - + - + + - + - +
Slx4-Flag - + + + + - + + + +
Dpb11-P
Dpb11
α-HA
Slx4-P
Slx4
α-FLAG
B
Rtt107-Flag/ Dpb11-HA
WCE
Flag-IP
Wt
slx4□
MMS - + - + - +
Dpb11-P
Dpb11
α-HA
Rtt107-P
Rtt107
α-FLAG
C
Rtt107-HA/ Dpb11-Flag
WCE
Flag-IP
MMS - + -/+ -/+
Rtt107-P
Rtt107
α-HA
D
Slx4-Flag/ Dpb11-HA
WCE
HA-IP
MMS - + +
Slx4-P
Slx4
α-FLAG
E
Wt cells
Rtt107-Flag
(MMS)
IP: Flag
incubate w/o phosphatase
incubate with phosphatase
Mix with lysate of slx4Δ Dpb11-HA
Mix with lysate of slx4Δ Dpb11-HA
IP: Rtt107-Flag (phosphorylated)
IP: Rtt107-Flag (unphosphorylated)
Dpb11
Rtt107-P
Rtt107
F
Dpb11
Mec1
Rtt107
Slx4

Figure 2-3

Figure 2.3. Slx4 Mediates a Phospho-Dependent Interaction between Rtt107 and Dpb11. (A) Co-IP experiment determining the effect of RTT107 deletion in the interaction between Slx4 and Dpb11. In our experimental conditions, Slx4 is not consistently detected in WCE, so cells lacking tagged Slx4 were also used in the experiment to confirm the specificity of the bands after IP. (B) Co-IP experiment determining the effect of SLX4 deletion in the interaction between Rtt107 and Dpb11. (C and D) Rtt107 and Slx4, respectively, bind Dpb11 in their slow-migrating phosphorylated form (−P). Because the band corresponding to Rtt107 exhibits a complete shift after MMS treatment, lysates from untreated and MMS-treated cells were mixed (±) prior to IP of Dpb11. (E) Cell culture expressing Rtt107-3XFlag was treated with 0.04% MMS for 2 hr, and Rtt107 was immunoprecipitated from protein extracts. Beads containing bound Rtt107 complex were divided into two halves: one half was treated with lambda phosphatase, and the other half was mock treated. The ability of each of the beads to interact with Dpb11 was determined by separately incubating them with native lysates from slx4Δ cells expressing Dpb11-3XHA. (F) A model for the assembly of the Rtt107-Slx4-Dpb11 complex. Dotted arrow indicates that Rtt107 is important, but not essential, for the Slx4-Dpb11 interaction.

Recruitment of Rtt107 and Slx4 to Dpb11 Requires Phosphorylation

I next decided to investigate whether phosphorylation is required for the interaction between Dpb11 and Rtt107-Slx4. First, we determined the phospho-status of Slx4 and Rtt107 when specifically bound to Dpb11, and we observed that both proteins are present in their slower migrating, phosphorylated state (Figures 2.3C and 2.3D). We then tested whether phosphorylation of the Rtt107-Slx4 complex is required for the interaction. Anti-Flag immunoprecipitates from wild-type cells harboring Flag-tagged Rtt107 (which coprecipitates stoichiometric amounts of Slx4) were treated with phosphatase or mock treated and then incubated with extracts from *slx4Δ* cells containing HA-tagged Dpb11 to assess their ability to bind Dpb11. As shown in Figure 2.3E, whereas the mock-treated immunoprecipitates bound Dpb11-HA, phosphatase-treated immunoprecipitates failed to show an Rtt107-Dpb11 interaction. In combination with our finding that Slx4 is required for the interaction, these results suggest that Slx4 phosphorylated by Mec1 binds Dpb11 directly (Figure 2.3F).

Phosphorylation of Slx4 by Mec1 Is Required for Its Interaction with Dpb11

To further test that phosphorylation of Slx4 by Mec1 is directly mediating the interaction of the Rtt107-Slx4 complex with Dpb11, we mutated canonical motifs for Mec1 phosphorylation (SQ/TQ) at the C-terminal region of Slx4, which shows conservation with human Slx4 (Figure 2.4A). Mutation of the three most conserved sites (3MUT), as judged by comparison with other closely related yeast species, reduced but did not abolish the mobility shift of Slx4 in response to MMS treatment

(Figure 2.4B). Likewise, interaction between Slx4 and Dpb11 was reduced but still detectable, suggesting the existence of redundant phosphorylation sites for mediating Dpb11-binding. Next, we mutated a total of seven residues to alanine, all located in the C-terminal region, resulting in the *SLX4-7MUT* mutant. As shown in Figure 2.4B, these mutations abolished the MMS-induced mobility shift of Slx4 and its ability to interact with Dpb11. *Slx4-7MUT* could still interact with Slx1 (Figure 2. S2A). These results, together with the finding that Slx4 is absolutely required for the binding of Rtt107 to Dpb11, strongly suggest that Slx4 phosphorylated by Mec1 binds Dpb11 directly.

The Slx4-Dpb11 Interaction Is Important for the Cellular Response to Replication Forks Blocked by DNA Alkylation

Slx4 and Rtt107 are believed to function as platforms to recruit proteins involved in DNA repair. As Dpb11 localizes to replication forks being engaged by Mec1 (Puddu et al., 2008), the interaction between Dpb11 and Slx4-Rtt107 identified here could represent a mechanism for the recruitment of fork repair factors to checkpoint engaged replication forks. To determine the functional importance of the interaction between Dpb11 and the Rtt107-Slx4 scaffolds, we integrated copies of the *SLX4* gene containing the 7MUT or 3MUT sequence at the endogenous genomic locus and tested for sensitivity to MMS (Figure 2.4C). Cells carrying *SLX4-3MUT* displayed only mild sensitivity. Strikingly, *SLX4-7MUT* cells displayed a significant sensitivity to MMS that was higher than that of *slx1Δ* cells but lower than that of *slx4Δ* cells. *SLX4-7MUT* cells were not sensitive to HU or CPT.

In addition, we have addressed the importance of Rtt107 phosphorylation by Mec1. We have mutated all SQ/TQ motifs in Rtt107 and could not observe any increased sensitivity to MMS (Figure 2.S2B), a result that sharply contradicts a previous report showing that mutation of these motifs leads to hypersensitivity to MMS (Rouse, 2004).

Next, I asked which of the replication stress-inducing agents can most efficiently induce the interaction between Rtt107-Slx4 with Dpb11. Of interest, Figure 2.4D shows that replication stress induced by MMS can most efficiently induce the interaction, in agreement with the selective MMS sensitivity exhibited by the *SLX4-7MUT* mutant. Replication stress induced by CPT did not promote detectable levels of interaction between Rtt107 and Dpb11, and replication stress induced by HU led to modest levels of interaction. Of interest, these results also reveal that, whereas double-strand breaks induced by phleomycin could strongly activate the DNA damage checkpoint, it could not promote detectable levels of interaction between Rtt107 and Dpb11 in G1 and led to limited interaction in G2-M. These results reveal that fork blockage generated by DNA alkylation during S phase most efficiently induces recruitment of Slx4-Rtt107 to Dpb11. I also monitored the temporal dynamics of Slx4-Dpb11 interaction following the release of cells from G1 into MMS-containing media and found that Slx4 only interacts with Dpb11 after Orc6 is phosphorylated (Figure 2.S2C), supporting that assembly of replication forks is required for Slx4 to interact with Dpb11. Taken together, the results suggest that the ability of Slx4 to bind to Dpb11 is critical for cells to properly respond to replication forks damaged by DNA alkylation.

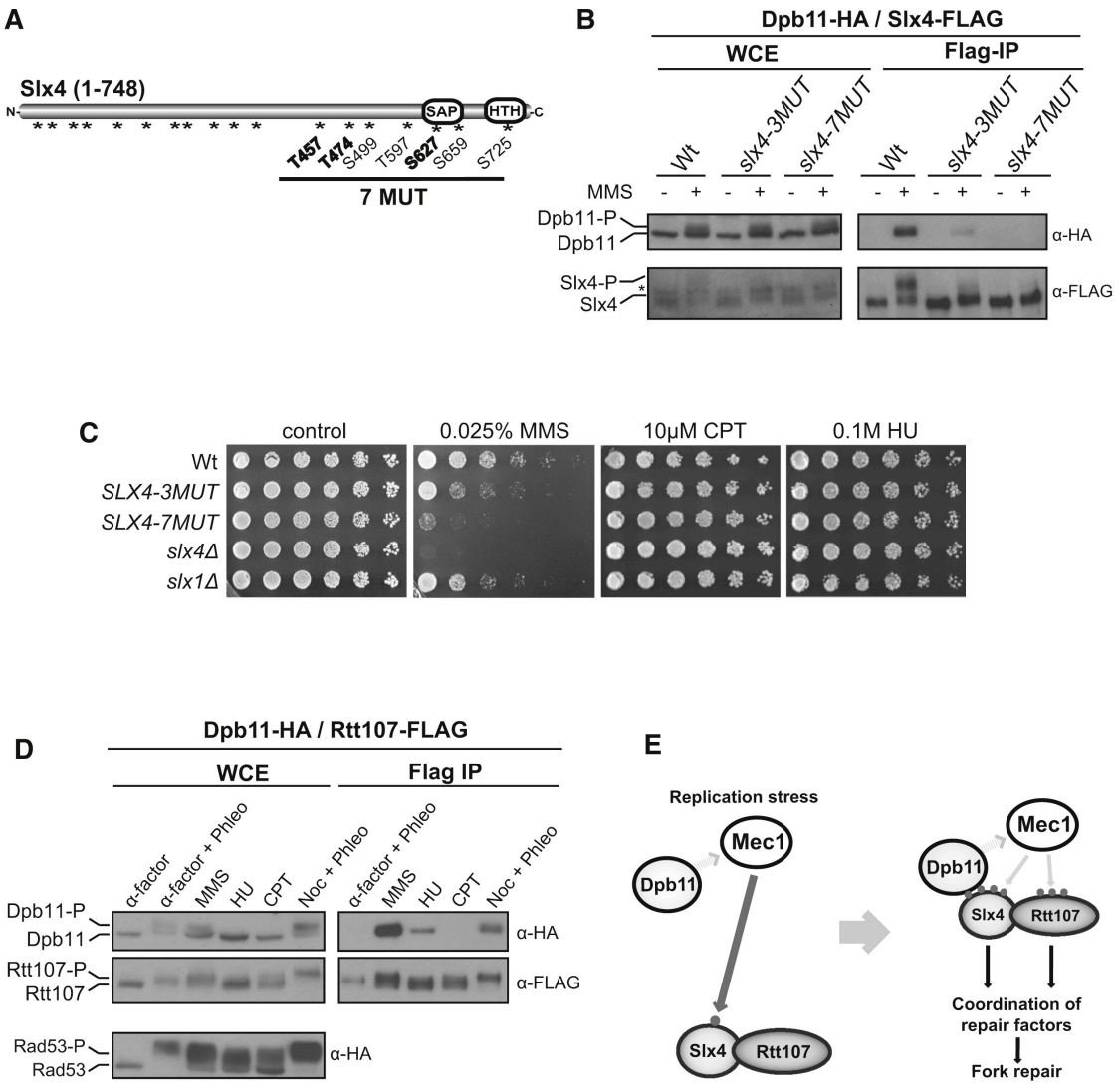

A
Slx4 (1-748)
N
SAP
HTH
C
T457
T474
S499
T597
S627
S659
S725
7 MUT
B
Dpb11-HA / Slx4-FLAG
WCE
Flag-IP
Wt
slx4-3MUT
slx4-7MUT
MMS
Dpb11-P
Dpb11
α-HA
Slx4-P
Slx4
α-FLAG
C
control
0.025% MMS
10μM CPT
0.1M HU
Wt
SLX4-3MUT
SLX4-7MUT
slx4Δ
slx1Δ
D
Dpb11-HA / Rtt107-FLAG
WCE
Flag IP
α-factor
α-factor + Phleo
MMS
HU
CPT
Noc + Phleo
Dpb11-P
Dpb11
Rtt107-P
Rtt107
Rad53-P
Rad53
α-HA
α-FLAG
E
Replication stress
Mec1
Dpb11
Slx4
Rtt107
Coordination of repair factors
Fork repair

Figure 2-4

Figure 2.4. Mutation of Canonical Mec1 Phosphorylation Sites in Slx4 Disrupts Its Interaction with Dpb11 and Compromises the Response to DNA Alkylation. (A) Schematic illustration of the Slx4 protein. Asterisks show the position of the 18 SQ/TQ motifs. The 7MUT mutant contains all seven indicated residues mutated to alanine. The 3MUT mutant contains mutation of the residues that are highly conserved among closely related yeast species (in bold). (B) Co-IP experiment determining the effect of 3MUT and 7MUT in Slx4 phospho-status and in the interaction with Dpb11. ∗, contaminating band. (C) Sensitivity of SLX4 mutants to MMS. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30°C. (D) Co-IP experiment from cells treated with the indicated drugs for 2 hr. Concentration of the drugs was as follow: α-factor (50 ng/ml), phleomycin (Phleo: 50 ug/ml), MMS (0.04%), hydroxyurea (HU: 100 mM), camptothecin (CPT: 5 ug/ml), nocodazole (Noc: 15 ug/ml). α-factor or nocodazole were added for 2 hr prior to the addition of phleomycin. As a control for the level of DNA damage checkpoint activation, cells containing HA-tagged Rad53 were subjected to identical treatments and analyzed by immunoblotting (bottom). (E) A model for the recruitment of Rtt107-Slx4 to the replication fork protein Dpb11 via Mec1-dependent phosphorylation. Gray dots represent Mec1-mediated phosphorylation.

DISCUSSION

How Mec1 regulates fork repair is not well understood. Here, we uncovered the mechanism by which Mec1 regulates the Slx4 and Rtt107 scaffolds, two critical coordinators of fork repair. I found that Mec1 recruits these scaffolds to the fork protein Dpb11 to mediate a response to replication stress. Dissection of the molecular basis of the interaction revealed that phosphorylation of Slx4 by Mec1 recruits the Slx4-Rtt107 unit to Dpb11. It remains to be shown whether phosphorylation of Slx4 leads to its enrichment precisely at stalled forks, as we were unable to perform ChIP analysis of Slx4 using a variety of epitope tags (data not shown). Of interest, Rtt107 appears to play an important but nonessential role in mediating the interaction of Slx4 with Dpb11, as deletion of RTT107 leads to a significant reduction of the interaction. Though the exact role of Rtt107 is still unclear, it could function to stabilize the complex together with Dpb11. In response to replication stress, Rtt107 and Slx4 are reciprocally required to become hyperphosphorylated by Mec1 (Figures 2.3A and 2.3B) (Roberts et al., 2006), suggesting that both proteins need to bind concurrently to Dpb11 in order to become stabilized at forks in close proximity to Mec1. The potential ability of Rtt107 to stabilize the Slx4-Dpb11 interaction would allow Slx4 to become hyperphosphorylated by Mec1, further enhancing the interaction of the Slx4-Rtt107 complex with Dpb11 and eventually leading to hyperphosphorylation of Rtt107 itself. This explanation is tempting, as Dpb11 is located close to Mec1 at stalled or damaged replication forks.

Our ability to disrupt the Slx4-Dpb11 interaction using the *SLX4-7MUT* mutant allowed us to establish that the interaction is important in the response to replication stress caused by DNA alkylation, which can damage replication forks. This raises interesting questions concerning how the recruitment of Rtt107-Slx4 to Dpb11 regulates fork repair. A logical explanation is that it may function to direct Slx4- or Rtt107-bound fork repair factors to Dpb11, which is located at damaged forks being engaged by the checkpoint. Indeed, I was able to detect a robust interaction between Dpb11 and the Slx1 endonuclease. Importantly, our results suggest that Slx4 and/or Rtt107 recruited to Dpb11 also play Slx1-independent functions, as the 7MUT mutant displays higher MMS sensitivity than slx1Δ cells. Additional structure-specific endonucleases not identified in our proteomic approach may also transiently interact with Dpb11 via Slx4, including the Rad1-Rad10 and Mus81-Mms4 endonucleases, whose human orthologs (XPF-ERCC1 and MUS81-EME1, respectively) were found to interact with human Slx4. In yeast, Rad1-Rad10 and Slx1 have been shown to interact with Slx4 in a mutually exclusive manner, but deletion of both nucleases does not result in MMS sensitivity as in *slx4Δ* cells (Flott et al., 2007). This argues that, in the absence of Slx1 and Rad1-Rad10, other nucleases may bind Slx4, potentially Mus81-Mms4. We were not able to detect strong interaction between Dpb11 and either the Rtt101 or Mms21 complexes by standard co-IP experiments of endogenously expressed proteins, probably because these represent more indirect interactions (via Slx4 and Rtt107). But it is interesting that Rtt101 has been shown to mediate replication over DNA damaged by MMS (Zaidi et al., 2008), which could relate to the phenotype exhibited by the *SLX4-7MUT*.

In conclusion, I provide a mechanism for the recruitment of the Slx4-Rtt107 scaffold unit to Dpb11 and propose a role for it in the coordination of fork repair (Figure 2.4E). In our proposed model, Mec1 is first recruited to stalled or damaged forks, where it becomes activated by Dpb11. Following activation, our data support that Mec1 then phosphorylates Slx4 to create a docking site for a direct interaction with Dpb11. Slx4 that interacts with Dpb11 is directly bound to Rtt107, allowing their concurrent recruitment to Dpb11, where they may coordinate the localization of various fork repair factors. Because Dpb11 is located at forks promoting Mec1 activation, I speculate that it represents an ideal anchor for the recruitment of repair factors that need to be specifically targeted to stalled or damaged replication forks. Together, our results reveal a role for Mec1 in the replication stress response and mechanistically connect DNA damage checkpoint signaling to factors involved in fork repair. The proteins participating in this recruiting mechanism are mostly conserved from yeast to humans, and remarkably, human Slx4 is also phosphorylated by ATR^{Mec1}, suggesting that our findings may represent a fundamental and conserved mechanism in fork repair. Recent analyses of Slx4 interactors in humans failed to identify TopBP1 (Fekairi et al., 2009 and Svendsen et al., 2009), but similarly to yeast, the Slx4-TopBP1 interaction may also require MMS treatment. Collectively, these findings improve our understanding of the role of DNA damage checkpoint in mediating the cellular response to damaged forks and open new directions for dissecting mechanisms of fork repair.

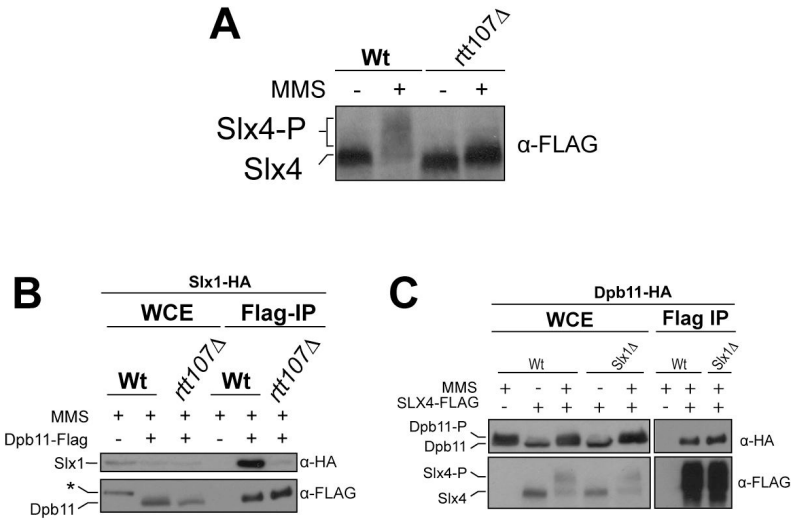

A
Wt
rtt107Δ
MMS - + - +
Slx4-P
Slx4
α-FLAG
B
Slx1-HA
WCE
Flag-IP
Wt
rtt107Δ
Wt
rtt107Δ
MMS + + + + + +
Dpb11-Flag - + + - + +
Slx1
α-HA
*
Dpb11
α-FLAG
C
Dpb11-HA
WCE
Flag IP
Wt
Slx1Δ
Wt
Slx1Δ
MMS + - + - + + + +
SLX4-FLAG - + + + + - + +
Dpb11-P
Dpb11
α-HA
Slx4-P
Slx4
α-FLAG

Figure 2-S1

Figure 2.S1 (related to Figure 2.3) (A) Slx4 is still phosphorylated upon MMS treatment in *rtt107Δ* cells, but to a lesser extent. Indicated cells expressing Slx4-Flag were treated with 0.04% MMS for 2 hours. Immunoblot was probed with anti-Flag antibody. (B) Slx1 physically interacts with Dpb11; and (C) Slx1 is not important for the interaction between Slx4 and Dpb11. (B and C) Cells with the indicated epitope-tagged proteins were treated with 0.04% MMS for 2 hrs. Protein extracts were immunoprecipitated with anti-Flag agarose resin and immunoblots were probed with anti-HA or anti-Flag antibodies. *: contaminating band.

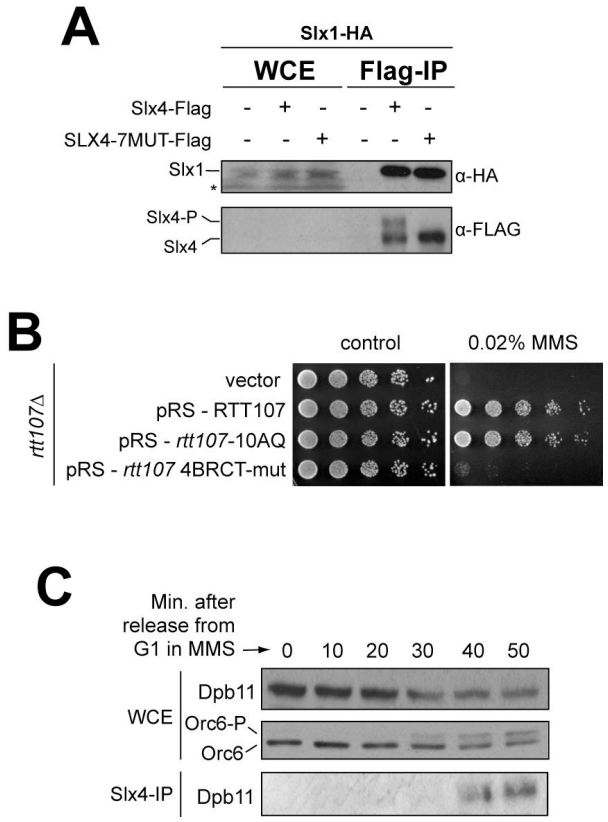

A
Slx1-HA
WCE
Flag-IP
Slx4-Flag - + - - + -
SLX4-7MUT-Flag - - + - - +
Slx1
*
α-HA
Slx4-P
Slx4
α-FLAG
B
control
0.02% MMS
rtt107Δ
vector
pRS - RTT107
pRS - rtt107-10AQ
pRS - rtt107 4BRCT-mut
C
Min. after release from G1 in MMS
0 10 20 30 40 50
WCE
Dpb11
Orc6-P
Orc6
Slx4-IP
Dpb11

Figure 2-S2

Figure 2.S2 (related to Figure 2.4). (A) *slx4-7MUT* still interacts with Slx1. Cells with the indicated epitope-tagged proteins were treated with 0.04% MMS for 2 hours. Protein extracts were immunoprecipitated with anti-Flag agarose resin and immunoblots were probed with anti-HA or anti-Flag antibodies. *: contaminating band. (B) Mutation of all 10 SQ/TQ motifs (10AQ) in Rtt107 does not lead to MMS sensitivity. Sensitivity of *rtt107Δ* cells expressing the indicated constructs. Four fold serial dilutions were spotted on plates and grown for 2 days at 30°C. 4BRCT-mut contains point mutations in four conserved tryptophan residues (mutated to phenylalanine) in the four n-terminal BRCT domains of Rtt107. (C) Slx4 interacts with Dpb11 following Orc6 phosphorylation. Cells expressing Slx4-Flag, Dpb11-HA and Orc6-HA were arrested in G1 and released in 0.04% MMS for the indicated time. For the Slx4-IP, protein extracts were immunoprecipitated with anti-Flag agarose resin and immunoblots were probed with anti-HA antibodies. Orc6 phosphorylation is an indicator of DNA replication initiation.

REFERENCES

Araki, H., Leem, S.H., Phongdara, A., and Sugino, A. (1995). Dpb11, which interacts with DNA polymerase II(epsilon) in Saccharomyces cerevisiae, has a dual role in S-phase progression and at a cell cycle checkpoint. Proc. Natl. Acad. Sci. USA 92, 11791–11795.

Branzei, D., Sollier, J., Liberi, G., Zhao, X., Maeda, D., Seki, M., Enomoto, T., Ohta, K., and Foiani, M. (2006). Ubc9- and mms21-mediated sumoylation counteracts recombinogenic events at damaged replication forks. Cell 127, 509–522.

Cobb, J.A., Bjergbaek, L., Shimada, K., Frei, C., and Gasser, S.M. (2003). DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1. EMBO J. 22, 4325–4336.

Coulon, S., Gaillard, P.H., Chahwan, C., McDonald, W.H., Yates, J.R., 3rd, and Russell, P. (2004). Slx1-Slx4 are subunits of a structure-specific endonuclease that maintains ribosomal DNA in fission yeast. Mol. Biol. Cell 15, 71–80.

Fekairi, S., Scaglione, S., Chahwan, C., Taylor, E.R., Tissier, A., Coulon, S., Dong, M.Q., Ruse, C., Yates, J.R., 3rd, Russell, P., et al. (2009). Human SLX4 is a Holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases. Cell 138, 78–89.

Flott, S., and Rouse, J. (2005). Slx4 becomes phosphorylated after DNA damage in a Mec1/Tel1-dependent manner and is required for repair of DNA alkylation damage. Biochem. J. 391, 325–333.

Flott, S., Alabert, C., Toh, G.W., Toth, R., Sugawara, N., Campbell, D.G.,

Haber, J.E., Pasero, P., and Rouse, J. (2007). Phosphorylation of Slx4 by Mec1 and Tel1 regulates the single-strand annealing mode of DNA repair in budding yeast. Mol. Cell. Biol. 27, 6433–6445.

Fricke, W.M., and Brill, S.J. (2003). Slx1-Slx4 is a second structure-specific endonuclease functionally redundant with Sgs1-Top3. Genes Dev. 17, 1768–1778.

Lopes, M., Cotta-Ramusino, C., Pellicioli, A., Liberi, G., Plevani, P., Muzi-Falconi, M., Newlon, C.S., and Foiani, M. (2001). The DNA replication checkpoint response stabilizes stalled replication forks. Nature 412, 557–561.

Luke, B., Versini, G., Jaquenoud, M., Zaidi, I.W., Kurz, T., Pintard, L., Pasero, P., and Peter, M. (2006). The cullin Rtt101p promotes replication fork progression through damaged DNA and natural pause sites. Curr. Biol. 16, 786–792.

Masumoto, H., Sugino, A., and Araki, H. (2000). Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast. Mol. Cell. Biol. 20, 2809–2817.

Mordes, D.A., Nam, E.A., and Cortez, D. (2008). Dpb11 activates the Mec1-Ddc2 complex. Proc. Natl. Acad. Sci. USA 105, 18730–18734.

Myung, K., Chen, C., and Kolodner, R.D. (2001). Multiple pathways cooperate in the suppression of genome instability in Saccharomyces cerevisiae. Nature 411, 1073–1076.

Navadgi-Patil, V.M., and Burgers, P.M. (2008). Yeast DNA replication protein Dpb11 activates the Mec1/ATR checkpoint kinase. J. Biol. Chem. 283, 35853–35859.

Ogiwara, H., Ui, A., Onoda, F., Tada, S., Enomoto, T., and Seki, M. (2006). Dpb11, the budding yeast homolog of TopBP1, functions with the checkpoint clamp in recombination repair. Nucleic Acids Res. 34, 3389–3398.

Puddu, F., Granata, M., Di Nola, L., Balestrini, A., Piergiovanni, G., Lazzaro, F., Giannattasio, M., Plevani, P., and Muzi-Falconi, M. (2008). Phosphorylation of the budding yeast 9-1-1 complex is required for Dpb11 function in the full activation of the UV-induced DNA damage checkpoint. Mol. Cell. Biol. 28, 4782–4793.

Roberts, T.M., Kobor, M.S., Bastin-Shanower, S.A., Ii, M., Horte, S.A., Gin, J.W., Emili, A., Rine, J., Brill, S.J., and Brown, G.W. (2006). Slx4 regulates DNA damage checkpoint-dependent phosphorylation of the BRCT domain protein Rtt107/Esc4. Mol. Biol. Cell 17, 539–548.

Roberts, T.M., Zaidi, I.W., Vaisica, J.A., Peter, M., and Brown, G.W. (2008). Regulation of rtt107 recruitment to stalled DNA replication forks by the cullin rtt101 and the rtt109 acetyltransferase. Mol. Biol. Cell 19, 171–180.

Rouse, J. (2004). Esc4p, a new target of Mec1p (ATR), promotes resumption of DNA synthesis after DNA damage. EMBO J. 23, 1188–1197.

Smolka, M.B., Albuquerque, C.P., Chen, S.H., and Zhou, H. (2007). Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. Proc. Natl. Acad. Sci. USA 104, 10364–10369.

Svendsen, J.M., Smogorzewska, A., Sowa, M.E., O'Connell, B.C., Gygi, S.P., Elledge, S.J., and Harper, J.W. (2009). Mammalian BTBD12/SLX4 assembles a Holliday junction resolvase and is required for DNA repair. Cell 138, 63–77.

Tercero, J.A., and Diffley, J.F. (2001). Regulation of DNA replication fork progression through damaged DNA by the Mec1/Rad53 checkpoint. Nature 412, 553–557.

Wang, H., and Elledge, S.J. (2002). Genetic and physical interactions between DPB11 and DDC1 in the yeast DNA damage response pathway. Genetics 160, 1295–1304.

Weinert, T.A., Kiser, G.L., and Hartwell, L.H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. 8, 652–665.

Zaidi, I.W., Rabut, G., Poveda, A., Scheel, H., Malmstro¨ m, J., Ulrich, H., Hofmann, K., Pasero, P., Peter, M., and Luke, B. (2008). Rtt101 and Mms1 in budding yeast form a CUL4(DDB1)-like ubiquitin ligase that promotes replication through damaged DNA. EMBO Rep. 9, 1034–1040.

Chapter 3[1]

DNA Repair Scaffolds Dampen Checkpoint Signaling by Counteracting the Rad9 Adaptor.

INTRODUCTION

In response to genotoxic stress, a transient arrest in cell-cycle progression enforced by the DNA-damage checkpoint (DDC) signalling pathway positively contributes to genome maintenance (Weinert and Hartwell, 1988). Because hyperactivated DDC signalling can lead to a persistent and detrimental cell-cycle arrest (Clerici *et al.* 2001, Pellicioli *et al.* 2001), cells must tightly regulate the activity of the kinases involved in this pathway. Despite their importance, the mechanisms for monitoring and modulating DDC signalling are not fully understood. Here I show that the DNA-repair scaffolding proteins Slx4 and Rtt107 prevent the aberrant hyperactivation of DDC signalling by lesions that are generated during DNA replication in *Saccharomyces cerevisiae*. On replication stress, cells lacking Slx4 or Rtt107 show hyperactivation of the downstream DDC kinase Rad53, whereas activation of the upstream DDC kinase Mec1 remains normal. An Slx4–Rtt107 complex counteracts the checkpoint adaptor Rad9 by physically interacting with Dpb11 and phosphorylated histone H2A, two positive regulators of Rad9-dependent Rad53 activation. A decrease in DDC signalling results from hypomorphic mutations in *RAD53* and *H2A* and rescues the hypersensitivity to replication stress of cells lacking Slx4 or Rtt107. I propose that the Slx4–Rtt107 complex modulates Rad53 activation by a competition-based mechanism that balances the engagement of Rad9

[1] Some of the experiments presented in this chapter were published in Ohouo et al., 2013.

at replication-induced lesions. Our findings show that DDC signalling is monitored and modulated through the direct action of DNA-repair factors.

MATERIALS AND METHODS

Yeast strains and plasmids

Strains generated in this study were derived either from MBS164 or MBS191 (both congenic to S288C) or W303 (where indicated). Unless indicated, all tags were inserted at the C terminus of the corresponding genes by homologous recombination at the genomic loci and were verified by western blotting. Tagged strains were assayed for sensitivity to MMS to ensure they behaved similarly to the wild-type strain. Sensitivity assays were independently confirmed in strains derived from freshly sporulated diploids. Standard cloning methods were used to generate the plasmids for this study. Plasmids containing domains of Dpb11 or Rtt107 tagged at the amino terminus with a PATH tag (2× protein A + TEV cleavage site + 6× His) (Smolka *et al*., 2006) were based on the pET21a vector (Novagen). HA-tagged full-length Rtt107, together with its native promoter, was cloned into pRS416 (Stratagene) to generate pMBS163. Wild-type alleles cloned into the pFA6a vector (Addgene) were linearized before integration into the respective endogenous loci. A pYES2/NT C vector (Invitrogen) containing full-length or C-terminal BRCTs of Rtt107 was used for overexpression. SLX4 and DPB11 constructs containing an ADH1 or a TDH3 promoter were generated by fusing the respective promoters (800 base pairs upstream of the start codon) to the corresponding open reading frame. The resultant PCR products were subsequently cloned into the pRS416 or pFA6a vector. All point mutations were generated by site-directed mutagenesis using either the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) or the PFU

Ultra II kit (Agilent). All yeast strains and plasmids used in this study are described in Tables 3.1 and 3.2.

Cell synchronization and genotoxin treatment

Yeast cells were grown in yeast peptone dextrose (YPD) or drop-out medium at 30 °C. Log phase cultures (optical density at 600 nm ≈ 0.3) were subjected to α-factor (0.5 μg ml^{-1}) or nocodazole (1.5 μg ml^{-1}) treatment for G1 or G2/M arrest, respectively. Cells were then washed and resuspended in warm medium containing the indicated genotoxin.

Western blotting and immunoprecipitation

For western blotting, about 50 mg frozen cell pellet was lysed by bead beating at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), Complete, EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP (Roche)). SDS loading buffer with 60 mM dithiothreitol (DTT) was added. Samples were separated by standard SDS-polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were detected using the following antibodies: anti-Rad53 (yc-19, 1:10,000, Santa Cruz Biotechnology), anti H2ApS129 (07-0745, 1:10,000, Millipore), anti-HA (12CA5, 1:10,000, Roche) and anti-Flag (M2, 1:5,000, Sigma) antibodies. For immunoprecipitation (IP), approximately 100 mg frozen cell pellet was lysed by bead beating at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, Complete, EDTA-free Protease Inhibitor Cocktail, 5 mM sodium fluoride and

10 mM β-glycerophosphate). After adjusting protein concentrations to about 6 mg ml^{-1}, inputs were aliquoted, and lysates were incubated with either anti-HA or anti-Flag agarose resin (Sigma) for 2–3 h at 4 °C. After three washes in lysis buffer, bound proteins were eluted with three resin volumes of SDS elution buffer (100 mM Tris-HCl, pH 8.0, and 1% SDS) for HA-IP or of Flag peptide (Sigma) solution (0.5 μg ml^{-1} in 100 mM Tris and 0.2% Tergitol) for Flag-IP. SDS loading buffer with DTT was added, and samples were analysed by western blotting with the indicated antibodies.

Pull-down with recombinant BRCT domain

Protein domains (for Dpb11 BRCT1/2, amino acids 1–270; and BRCT3/4, amino acids 271–582) containing an N-terminal PATH tag (see Yeast strains and plasmids) were expressed in Escherichia coli, bound to human IgG–agarose resin (GE Healthcare) and then used as bait for pull-downs from yeast lysates as previously described (Smolka *et al*., 2006).

SILAC labelling of yeast

For mass spectrometry experiments, cells were grown in (−)Arg (−)Lys drop-out medium ('light' version complemented with normal arginine and lysine; 'heavy' version complemented with lysine 13C6, 15N2 and arginine 13C6, 15N4) for at least five generations.

Purification of phosphopeptides by immobilized metal-ion affinity chromatography (IMAC)

For the purification of Rad53 phosphopeptides, approximately 0.6 g cell pellet from wild-type (grown in heavy medium) or *slx4Δ* (grown in light medium) strains carrying Rad53–HA was lysed by bead beating at 4 °C in 4 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, Complete, EDTA-free Protease Inhibitor Cocktail, 5 mM sodium fluoride and 10 mM β-glycerophosphate). Lysates were incubated with anti-HA–agarose resin (Sigma) for 4 h at 4 °C. After three washes with lysis buffer, bound proteins were eluted with three resin volumes of elution buffer (100 mM Tris-HCl, pH 8.0, and 1% SDS). Eluted proteins from light or heavy medium were mixed together, reduced, alkylated and precipitated. Proteins were resuspended in a solution of 2 M urea and 12.5 mM Tris-HCl, pH 8.0, and digested with trypsin for 16 h at 37 °C. Phosphopeptides were enriched using an 'in-house' IMAC column, then eluted with 10% ammonia and 10% acetonitrile and dried in a SpeedVac evaporator.

Phosphoproteome analysis

Approximately 0.6 g cell pellet from wild-type (grown in light medium) and *slx4Δ* (grown in heavy medium) strains was lysed by bead beating at 4 °C in 4 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, Complete, EDTA-free Protease Inhibitor Cocktail (Roche), 5 mM sodium fluoride and 10 mM β-glycerophosphate). Protein lysates were denatured in 1% SDS, reduced, alkylated and then precipitated with three volumes of a solution containing 50%

acetone and 50% ethanol. Proteins were solubilized in a solution of 2 M urea, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl, and then TPCK-treated trypsin was added. Digestion was performed overnight at 37 °C, and then trifluoroacetic acid and formic acid were added to a final concentration of 0.2%. Peptides were desalted with a Sep-Pak C18 column (Waters), dried in a SpeedVac evaporator and resuspended in 1% acetic acid. Phosphopeptides were enriched by IMAC as previously described (Albuquerque *et al.* 2008, Smolka *et al.* 2007, Smolka *et al.* 2005) reconstituted in 85 µl solution containing 80% acetonitrile and 1% formic acid, and fractionated by hydrophilic interaction liquid chromatography (HILIC) as previously described (Albuquerque *et al.* 2008), before analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS). More than 3,570 phosphopeptides were identified or quantified, and phosphopeptides containing a phosphorylation site known to be a Mec1 target or a target of Rad53-dependent phosphorylation (Smolka *et al.* 2007, Chen *et al.* 2010) were selected.

Mass spectrometry analysis

IMAC elutions or HILIC fractions were dried in a SpeedVac evaporator, reconstituted in 0.1% trifluoroacetic acid and analysed by LC-MS/MS using a 125 µM ID capillary C18 column and an Orbitrap XL mass spectrometer coupled with an Eksigent nanoflow system. Database searching was performed using the SORCERER system (Sage-N Research) running the program SEQUEST. After searching a target-decoy budding yeast database, results were filtered either based on probability score to achieve a 1% false positive rate or manual inspection.

Quantification of heavy/light peptide isotope ratios was performed using the Xpress program as previously described (Smolka *et al.* 2007).

Chromatin immunoprecipitation (ChIP)

Cultures were grown in YPD to an optical density at 600 nm ≈ 0.3, arrested in G1 with α-factor for 2 h and released in the presence of 200 mM hydroxyurea (HU) for 1 h. Cultures were formaldehyde-crosslinked (1% final concentration) for 20 min followed by quenching with 125 mM glycine. Protein–DNA complexes were immunoprecipitated with a yeast-specific anti-phospho-histone (H2ApS129) antibody (07-745, Millipore). Quantitative PCR was performed with the purified DNA from the immunoprecipitate as previously described (Petesch and Lis, 2008) using pairs of primers designed to amplify the genome sequences shown in Figure 3.4c. The primer sequences were as follows:

39.3Kb_for, 5′CAAGTGGATTGAGGCCACAGCA-3′,

39.3Kb_rev, 5′-CCGGACAGTACATGAAACTGGACA-3′;

43.1Kb_for, 5′-TCAAGGTGGCTTGATGATCGCC-3′,

43.1Kb_rev, 5′-CACCTCCAATCTGCTTCAAGTTTGGC-3′;

47.3Kb_for, 5′-TATCTTGCGGGCCTTTCGTGTC-3′, and

47.3Kb_rev, 5′-GGGAGATTCCATTTCCGCACCA-3′.

Table 3.1 Yeast strains used in this study

Strain No.	Relevant genotype
MBS1 = DDY1810	*MATa, leu2, ura3-52, trp1, prb1-1122, pep4-3, pre1-451, 6xHis-3xHA_RAD9::KanMX6*
MBS164	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3*
MBS191	*ura3-52/ura3-52, trp1-63/trp1-63, his3-200/his3-200*
MBS223	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::KanMX6, mrc1Δ:URA3*
MBS224	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, rtt107Δ:KanMX6*
MBS294	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::KanMX6, rad9Δ:URA3*
MBS313	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::KanMX6, rtt107Δ:URA3*
MBS336	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2*
MBS373	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xhis3-3xHA::LEU2, mrc1Δ:URA3, rtt107Δ:KanMX6*
MBS444	*ura3-52 trp1-63 his3-200, rtt107Δ:HIS3 (Spore)*
MBS448	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA::LEU2*
MBS571	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA::LEU2, SLX4_6xHis-3FLAG::KanMX6*
MBS574	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, SLX4_6xHis-3xFLAG::KanMX6*
MBS607	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2, slx4Δ:KanMX6*
MBS611	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, slx4Δ:URA3*
MBS626	*MATa, ura3-52, trp1-63, his3-200, bar1Δ:HIS3*
MBS943 = yDD_705 (W303)	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 GAL+ psi+ ssd1-d2 RAD5+*
MBS944 = yDD_1793 (W303)	*MATa hta1-S129A hta2-S129A leu2-3, 112 his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100 RAD5+*
MBS957 (W303)	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 GAL+ psi+ ssd1-d2 RAD5+, rtt107Δ:HIS3*
MBS958 (W303)	*MATa hta1-S129A hta2-S129A leu2-3, 112 his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100 RAD5+, rtt107Δ:HIS3*

MBS961 (W303)	*MATa hta1-S129A hta2-S129A leu2-3, 112 his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100 RAD5+, slx4:ΔHIS3*
MBS975 (W303)	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 GAL+ psi+ ssd1-d2 RAD5+, SLX4_6xHis-3xFLAG::KanMX6*
MBS976 (W303)	*MATa hta1-S129A hta2-S129A leu2-3, 112 his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100 RAD5+, SLX4_6xHis-3xFLAG::KanMX6*
MBS982	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA_LEU2, SLX4_6xHis-3xFLAG::KanMX6, mrc1Δ:URA3*
MBS1002 (W303)	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 GAL+ psi+ ssd1-d2 RAD5+, slx4Δ:HIS3*
MBS1187	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11-3xFLAG::LEU2, RAD9-3xHA::KanMX6*
MBS1197	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD9_3xHA::KanMX6*
MBS1207	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11-3xFLAG::LEU2, Rad9_3xHA::KanMX6, slx4Δ:URA3*
MBS1230	*MATa, ura3-52, trp1-63, his3-200, slx4Δ:HIS3, bar1Δ:URA3*
MBS1237	*MATa, ura3-52, trp1-63, his3-200, bar1Δ:URA3, mrc1Δ:KanMX6*
MBS1278	*MATa, ura3-52, trp1-63, his3-200, slx4Δ:HIS3, bar1Δ:URA3, mrc1Δ:KanMX6*
MBS1301	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA::LEU2, slx4::slx4-S486A_6xHis-3xFLAG::KanMX6*
MBS1302	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA::LEU2, slx4::slx4-7MUT_6xHis-3xFLAG::KanMX6*
MBS1307	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2, slx4Δ:URA3*
MBS1309	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2, slx4Δ:KanMX6, rad9Δ:URA3*
MBS1313	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2, slx4::slx4-7MUT_6xHis-3xFLAG::KanMX6*
MBS1343	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2, slx4Δ:KanMX6, mrc1Δ:URA3*
MBS1350	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA::LEU2, slx4::slx4-7MUT-S486A_6xHis-3xFLAG::KanMX6*
MBS1437	*MATa, ura3-52, trp1-63, his3-200, RAD53_6xHis-3xFLAG::KanMX6 (Spore)*
MBS1438	*MATa, ura3-52, trp1-63, his3-200, RAD53_6xHis-3xFLAG::KanMX6, slx4Δ:HIS3 (Spore)*
MBS1439	*MATa, ura3-52, trp1-63, his3-200, rad53-R70A_6xHis-3xFLAG::KanMX6 (Spore)*

MBS1440	*MATta, ura3-52, trp1-63, his3-200, rad53-R605A_6xHis-3xFLAG::KanMX6 (Spore)*
MBS1441	*ura3-52, trp1-63, his3-200, rad53-R70A_ 6xHis-3xFLAG::KanMX6, slx4Δ:HIS3 (Spore)*
MBS1442	*MATa, ura3-52, trp1-63, his3-200, rad53-R605A_ 6xHis-3xFLAG::KanMX6, slx4Δ:HIS3 (Spore)*
MBS1448 (W303)	*MATa hta1-S129A hta2-S129A leu2-3, 112 his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100 RAD5+, RAD53_6xHis-3xHA::KanMX6*
MBS1452	*MATa, ura3-52, trp1-63, his3-200, RAD53_6xHis-3xFLAG::KanMX6, bar1Δ:URA3*
MBS1453	*MATa, ura3-52, trp1-63, his3-200, RAD53_6xHis-3xFLAG::KanMX6::KanMX6, slx4Δ:HIS3, bar1Δ:URA3*
MBS1454	*MATa, ura3-52, trp1-63, his3-200, rad53-R605A_ 6xHis-3xFLAG::KanMX6, bar1Δ:URA3*
MBS1464	*MATa, ura3-52, trp1-63, his3-200, rad53-R605A_ 6xHis-3xFLAG::KanMX6, slx4Δ:HIS3, bar1Δ:URA3*
MBS1483 (W303)	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 GAL+ psi+ ssd1-d2 RAD5+, RAD53_6xHis-3xHA::KanMX6*
MBS1489 (W303)	*MATa hta1-S129A hta2-S129A leu2-3, 112 his3-11, 15 ade2-1 ura3-1 trp1-1 can1-100 RAD5+, RAD53_6xHis-3xHA::KanMX6, rtt107Δ:HIS3*
MBS1493	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::NatMX4, rad9Δ:URA3*
MBS1503 (W303)	*MATa ade2-1 trp1-1 can1-100 leu2-3, 112 his3-11, 15 ura3-1 GAL+ psi+ ssd1-d2 RAD5+, RAD53_6xHis-3xHA::KanMX6, rtt107Δ:HIS3*
MBS1508	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::NatMX4, rad9::RAD9_3xHA::KanMX6*
MBS1510	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::NatMX4, rad9:: rad9-K1088M_3xHA::KanMX6*
MBS1514	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::NatMX4, rad9::RAD9_3xHA::KanMX6, rtt107Δ:URA3*
MBS1515	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::NatMX4, rad9::RAD9_3xHA::KanMX6, rtt107Δ:URA3*
MBS1516	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::NatMX4, rad9:: rad9-K1088M_3xHA::KanMX6, rtt107Δ:URA3*
MBS1517	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::NatMX4, rad9:: rad9-K1088M_3xHA::KanMX6, rtt107Δ:URA3*
MBS1529	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA::LEU2, slx4Δ:NatMX4*

MBS1551	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA::LEU2, RAD9_6xHis-3FLAG::KanMX6*
MBS1554	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, DPB11_6xHis-3xHA::LEU2, slx4Δ:NatMX4, RAD9_6xHis-3xFLAG::KanMX6*
MBS1579	*ura3-52, trp1-63, his3-200*
MBS1580	*ura3-52, trp1-63, his3-200, slx4Δ:HIS3*
MBS1581	*ura3-52, trp1-63, his3-200, rad53-R605A::KanMX6*
MBS1582	*ura3-52, trp1-63, his3-200, slx4Δ:HIS3, rad53-R605A::KanMX6*
MBS1583	*ura3-52, trp1-63, his3-200, slx4Δ:HIS3, rad53-R605A::KanMX6*
MBS1591	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3HA::LEU2, slx4::slx4-S486A _6xHis -3xFLAG::KanMX6*
MBS1592	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3HA::LEU2, slx4::slx4-7MUT-S486A_6xHis-3xFLAG::KanMX6*
MBS1624	*MATa,ura3-52,leu2Δ1,trp1Δ63,his3Δ200,lys2ΔBgl,hom3-10, ade2Δ1,ade8,arg4Δ,sml1::TRP1,bar1::HIS3,Rad53-6xHis-3xHA-LEU2,slx1::URA3*
MBS1625	*MATa,ura3-52,leu2Δ1,trp1Δ63,his3Δ200,lys2ΔBgl,hom3-10, ade2Δ1,ade8,arg4Δ,sml1::TRP1,bar1::HIS3,Rad53-6xHis-3xHA-LEU2,rad1::KanMX6*
MBS1629	*MATa,ura3-52,leu2Δ1,trp1Δ63,his3Δ200,lys2ΔBgl,hom3-10, ade2Δ1,ade8,arg4Δ,sml1::TRP1,bar1::HIS3,Rad53-6xHis-3xHA-LEU2,slx1::URA3,rad1::KanMX6*
MBS1649	*MATa,ura3-52,leu2Δ1,trp1Δ63,his3Δ200,lys2ΔBgl,hom3-10, ade2Δ1,ade8,arg4Δ,sml1::TRP1,bar1::HIS3,Rad53-6xHis-3xHA-LEU2,dot1::NatMX4*
MBS1723	*MATa,ura3-52,trp1-63,his3-200,bar1::HIS3,Rad53-3xHA-TRP1*
MBS1755	*MATa,ura3-52,trp1-63,his3-200,bar1::HIS3,dot1::NatMX4,Rad53-3xHA- TRP1*
MBS1867	*MATa,ura3-52,trp1-63,his3-200,bar1::HIS3,Rad9-6xHisx3FLAG- KanMX6*
MBS1972	*MATa,ura3-52,trp1-63,his3-200,bar1::HIS3,Rad9-3xFLAG-TRP1, pADH1-Slx4-13xMyc-KanMX6*
MBS1974	*MATa,ura3-52,trp1-63,his3-200,bar1::HIS3,Rad9-3xFLAG-TRP1, pADH1-slx4-S486A-13xMyc-KanMX6*
MBS1989	*MATa,ura3-52,trp1-63,his3-200,bar1::HIS3,pADH1-Slx4-13xMyc-NatMX4,Rad53-3xHA-TRP1*
MBS2004	*MATa,ura3-52,trp1-63,his3-200,bar1::HIS3,pADH1-Slx4-13xMyc-NatMX4,Rad53-3xHA-TRP1,dot1::KanMX6*

Table 3.2 Plasmids used in this study

Plasmid No.	Vector	Gene	Mutation	Tag
pMBS32 = HZE1210	pFA6a	*RAD53*	-	-
pMBS38 = HZE1267	pFA6a	*RAD53*	-	FLAG
pMBS39 = HZE1268	pFA6a	*rad53*	R70A	FLAG
pMBS40 = HZE1269	pFA6a	*rad53*	R605A	FLAG
pMBS65	pYES2/NT C	-	-	HA
pMBS148	pRS416	-	-	-
pMBS163	pRS416	*RTT107*	-	HA
pMBS166	pYES-ntc	*RTT107*	-	HA
pMBS175	pFA6a	*RAD9*	-	HA
pMBS197	pYES2/NT C	*RTT107*(aa818-1070)	-	HA
pMBS218	pFA6a	*SLX4*	-	FLAG
pMBS220	pFA6a	*slx4*	T457A, T474A, S499A T597A, S627A, S659A, S725A	FLAG
pMBS249	pET21a	*RTT107*(aa750-1070)	-	PATH
pMBS250	pET21a	*rtt107*(aa750-1070)	R884E	PATH
pMBS261	pRS416	*rtt107*	R884E	HA
pMBS297	pET21a	*DPB11*(aa1-270)	-	PATH
pMBS298	pET21a	*DPB11*(aa271-582)	-	PATH
pMBS327	pFA6a	*slx4*	S486A	FLAG
pMBS340	pFA6a	*slx4*	T457A, T474A, S499A, T597A, S627A, S659A, S725A, S486A	FLAG
pMBS349	pFA6a	*rad9*	K1088M	HA
pMBS362	pFA6a	*rad53*	R605A	-
pMBS375	pYES2/NT C	*rtt107*(aa818-1070)	R884E	HA
pMBS401	pRS416	*pADH1-SLX4**	-	Myc
pMBS402	pRS416	*pTDH3-SLX4**	-	Myc
pMBS406	pRS416	*pTDH3-slx4**	S486A	Myc
pMBS414	pRS416	*pADH1-DPB11**	-	HA
pMBS435	pFA6a	*pADH1-SLX4**	-	Myc
pMBS436		*pADH1-slx4**	S486A	Myc

* Cloned with the ADH1 or the TDH3 promoter.

RESULTS

Slx4 Counteracts Rad9-dependent Rad53 Activation

Slx4 is an evolutionarily conserved DNA-repair scaffolding protein that is important for the cellular response to exogenous DNA-damaging agents (Fekairi *et al.* 2009, Svendsen *et al.* 2009, Muñoz *et al.* 2009, Fricke and Brill 2003) and mutations in human *SLX4* were recently linked to Fanconi anaemia (Stoepker *et al.* 2011, Kim *et al.* 2011). In *S. cerevisiae* (budding yeast), cells that lack *SLX4* (*slx4*Δ cells) are highly sensitive to methyl methanesulphonate (MMS) (Fricke and Brill 2003), a DNA-alkylating agent that blocks replication and induces the DDC pathway. While investigating the activation status of the *S. cerevisiae* DDC kinase Rad53 in *slx4*Δ cells, we noted that MMS treatment leads to hyperphosphorylation, and thus hyperactivation, of Rad53 compared with in wild-type cells (Figure 3.1a and Supplementary Figure 3.1a), which is consistent with a previous report (Roberts *et al.* 2006). However, the phosphorylation of histone H2A (also known as Hta1 and Hta2), a substrate of the upstream DDC kinase Mec1, at serine 129 (here referred to as $H2A^{pS129}$) was not increased in *slx4*Δ cells (Figure 3.1a, lower panel). These results suggest that the hyperactivation of Rad53 in *slx4*Δ cells is not caused by increased damage-induced Mec1 signalling but by improper downstream regulation of Rad53 activation. To test this possibility, we compared the phosphoproteome of wild-type and *slx4*Δ cells after MMS treatment, using quantitative mass spectrometry. Although most of the detected Mec1 targets were phosphorylated to the same extent in both cell types, Rad53-dependent phosphorylation was significantly higher in *slx4*Δ cells than in wild-type cells (Supplementary Figure

3.1b), further supporting the idea that Slx4 has a role in specifically blocking Rad53 hyperactivation. Because the activation of Rad53 in response to MMS mostly depends on the checkpoint adaptor Rad9 (Figure 3.1b and Supplementary Figure 3.2), Slx4 probably counteracts Rad9-dependent Rad53 activation. To test whether the sensitivity of *slx4*Δ cells to MMS is caused mostly by aberrant Rad53 hyperactivation, I used hypomorphic alleles of *rad53* that result in lower Rad53 activation, reasoning that these alleles would rescue the MMS sensitivity of *slx4*Δ cells. Rad53 has two FHA domains, which bind to phosphorylated Rad9 in a redundant manner and mediate Rad53 activation (Schwartz *et al.* 2002) (Figure 3.1c). Mutations in the FHA2 domain promote a stronger reduction in MMS-induced Rad53 activation than do mutations in the FHA1 domain (Schwartz *et al.* 2003). Whereas a mutation (R70A, in which arginine is substituted with alanine at amino acid 70) in the FHA1 domain of Rad53 had no effect on the MMS sensitivity of *slx4*Δ cells, a mutation (R605A) in the FHA2 domain reduced the MMS sensitivity of *slx4*Δ cells (Figure 3.1d). Consistent with our hypothesis that Rad53 hyperactivation is the cause of the MMS sensitivity of *slx4*Δ cells, mutation of the FHA2 domain resulted in a decrease in Rad53 activation in *slx4*Δ cells to a level similar to that of wild-type cells (Figure 3.1e). Collectively, these results suggest that Slx4 has a crucial role in preventing excessive Rad9-dependent activation of Rad53 (Figure 3.1f). The levels of MMS used here require that cells pass through the S phase of the cell cycle for Rad53 to become active (Tercero *et al.* 2003); therefore, our results suggest that Slx4 counteracts the Rad9–Rad53 pathway in response to replication-induced lesions. The finding that combined deletion of the *SLX1* and *RAD1* genes, which

encode nucleases that are known to associate with Slx4, leads to lower MMS sensitivity and Rad53 activation than in *slx4*Δ cells (Supplementary Figure 3.4) supports a nuclease-independent function for Slx4 during the cellular response to MMS-induced replication stress.

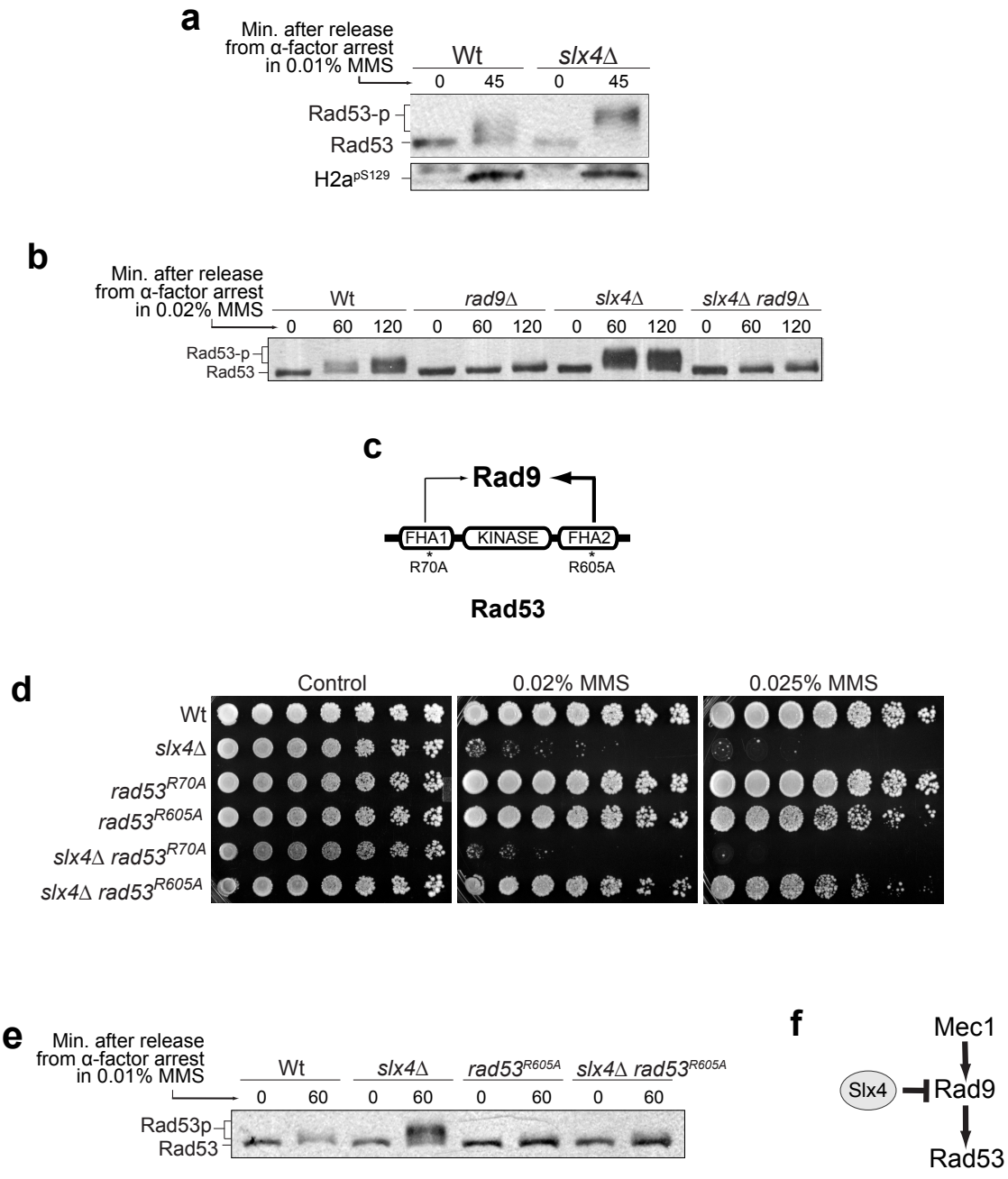

a
Min. after release from α-factor arrest in 0.01% MMS
Wt
slx4Δ
0
45
0
45
Rad53-p
Rad53
H2apS129
b
Min. after release from α-factor arrest in 0.02% MMS
Wt
rad9Δ
slx4Δ
slx4Δ rad9Δ
0 60 120 0 60 120 0 60 120 0 60 120
Rad53-p
Rad53
c
Rad9
FHA1
KINASE
FHA2
R70A
R605A
Rad53
d
Control
0.02% MMS
0.025% MMS
Wt
slx4Δ
rad53R70A
rad53R605A
slx4Δ rad53R70A
slx4Δ rad53R605A
e
Min. after release from α-factor arrest in 0.01% MMS
Wt
slx4Δ
rad53R605A
slx4Δ rad53R605A
0 60 0 60 0 60 0 60
Rad53p
Rad53
f
Mec1
Slx4
Rad9
Rad53

Figure 3-1

Figure 3.1. a, Western blot showing phosphorylation of haemagglutinin (HA)-tagged Rad53 and histone H2ApS129 after MMS treatment. b, Western blot showing the phosphorylation status of Rad53–HA in the indicated strains after MMS treatment. c, Schematic representation of the Rad53 protein and its interaction with Rad9. d, MMS sensitivity assay of strains containing the indicated Flag-tagged mutant Rad53 proteins. Similar results were obtained with strains containing untagged Rad53 (Supplementary Figure 3.3). e, Western blot showing the phosphorylation status of Rad53–Flag in the indicated strains after MMS treatment. f, Model for the role of Slx4 in uncoupling Rad53 activation from Mec1 signalling, through counteracting Rad9. Rad53-p, phosphorylated Rad53; WT, wild type.

Slx4 Binding to Dpb11 Counteracts the Dpb11-Rad9 Interaction and Rad53 Activation.

We recently reported that on replication stress, Slx4 binds to Dpb11 (Ohouo *et al.* 2010), a replication factor that is involved in DDC activation (Navadgi-Patil *et al.* 2008, Mordes *et al.* 2008). Because Dpb11 binds to Rad9 and positively regulates Rad9-dependent Rad53 activation (Granata *et al.* 2010, Pfander and Diffley 2011), we assessed whether the Slx4–Dpb11 interaction has a role in counteracting Rad53 activation during MMS treatment. We previously showed that phosphorylation of Slx4 by Mec1 mediates the interaction of Slx4 with Dpb11 and that an Slx4 mutant lacking seven Mec1 consensus phosphorylation sites (Slx4-7MUT) cannot stably interact with Dpb11 (Ohouo *et al.* 2010). Rad53 is hyperactivated in *slx4-7MUT* cells (Figure 3.2a), supporting a model in which the Slx4–Dpb11 interaction is important for preventing Rad53 hyperactivation. Next, we tested whether the Slx4–Dpb11 interaction inhibits the ability of Rad9 to bind to Dpb11 in wild-type and *slx4Δ* cells. Deletion of SLX4 leads to a significant increase in the MMS-induced interaction between Dpb11 and Rad9 (Figure 3.2b and Supplementary Figure 3.5), suggesting that Slx4 and Rad9 compete for Dpb11 binding. Dpb11 contains two pairs of BRCT domains, which bind to phosphorylated motifs. We found that recombinant BRCT domains 1 and 2 (BRCT1/2) of Dpb11 can bind phosphorylated Slx4 and phosphorylated Rad9 from MMS-treated S. cerevisiae lysates (Figure 3.2c). This finding is consistent with a model in which Slx4 and Rad9 compete for BRCT1/2 binding.

Dpb11 BRCT1/2 was previously shown to interact directly with cyclin-dependent kinase (CDK)-dependent phosphorylation sites in Sld3, thereby initiating DNA replication (Tanaka *et al.* 2007, Zegerman and Diffley 2007). To better understand the mechanism of the Slx4–Dpb11 interaction, we searched for a CDK-targeting motif in Slx4 that resembles the Dpb11-binding region in Sld3. Notably, Slx4 contains proline-directed phosphorylation sites that align well with serine 600 (S600) and S622 of Sld3, which are important for the Dpb11–Sld3 interaction (Tanaka *et al.* 2007, Zegerman and Diffley 2007) (Supplementary Figure 3.6). We tested the importance of the proline-directed sites within the Sld3-like region of Slx4 and found that S486 of Slx4 is crucial for the MMS-induced interaction with Dpb11 (Figure 3.2d). In addition, three canonical Mec1 phosphorylation sites (S/T-Q sites) that are important for mediating a robust Slx4–Dpb11 interaction (Ohouo *et al.* 2010) are located in or near the Sld3-like Dpb11-binding region in Slx4. I conclude that the Slx4–Dpb11 interaction is mediated by the coordinated action of both Mec1 and a proline-directed kinase at a motif that is probably targeted by BRCT1/2 of Dpb11. Although we detected residual binding between Dpb11 and Slx4-7MUT, we did not detect an interaction between Dpb11 and Slx4 S486A, suggesting that the phosphorylation of S486 has a more important role in mediating the Slx4–Dpb11 interaction than the Mec1 consensus phosphorylation sites. This is also supported by the finding that slx4 S486A leads to Rad53 hyperactivation (Figure 3.2e) and to a higher MMS sensitivity than does slx4-7MUT (Figure 3.2f). Interestingly, previous reports showed that proline-directed sites in Rad9 are also important for the Dpb11–Rad9 interaction (Granata *et al.* 2010, Pfander and Diffley

2011) (Supplementary Figure 3.7). To further test the model that Slx4 antagonizes Rad53 activation by binding to Dpb11 and outcompeting Rad9, I overexpressed Slx4 using an *ADH1* or a *TDH3* promoter and monitored different steps of checkpoint activation. In an early step in checkpoint activation, Rad9 assembles into a ternary complex with Dpb11 and Mec1 and is hyperphosphorylated by Mec1 (Pfander and Diffley 2011). We first monitored the phosphorylation status of Rad9 in cells expressing Slx4 from its endogenous promoter and in cells overexpressing Slx4. Overexpression of wild-type Slx4 but not S486A mutated Slx4 significantly inhibited the MMS-induced hyperphosphorylation of Rad9 (Figure 3.2g), as shown by the strong decrease in the slower migrating band at 45 and 60 min after release from α-factor arrest. We then monitored the effect of Slx4 overexpression on Rad53 activation in response to MMS treatment (Supplementary Figure 3.8a). Although overexpression of Slx4 leads to a small but consistent decrease in Rad53 activation early in the response, we did not observe the same effect at later time points. I speculated that after 40 min, the Rad53 activation that was observed in Slx4-overexpressing cells was being mediated by a parallel mechanism based on Dot1-mediated histone H3K79 methylation, which can promote the recruitment of Rad9 to lesion sites independently of Dpb11 (Puddu, F. *et al.* 2008). I therefore monitored Rad53 activation in cells lacking *DOT1* and found that Slx4 overexpression significantly reduces Rad53 activation also at later time points (Figure 3.2h); this effect depends on S486 of Slx4 (Supplementary Figure 3.8b, c). Next, I determined whether Slx4 overexpression specifically disrupts the Rad9–Dpb11 interaction. I detected hyperphosphorylated Rad9 in a Dpb11 pull-down from cells expressing

Slx4 from its endogenous promoter, but I did not detect Rad9 in a Dpb11 pull-down using cells that overexpressed Slx4 (Figure 3.2i). This effect was specific for S486 of Slx4. These findings support our model that Slx4 counteracts DDC signalling by binding to Dpb11 and preventing its stable interaction with Rad9 (Figure 3.2j).

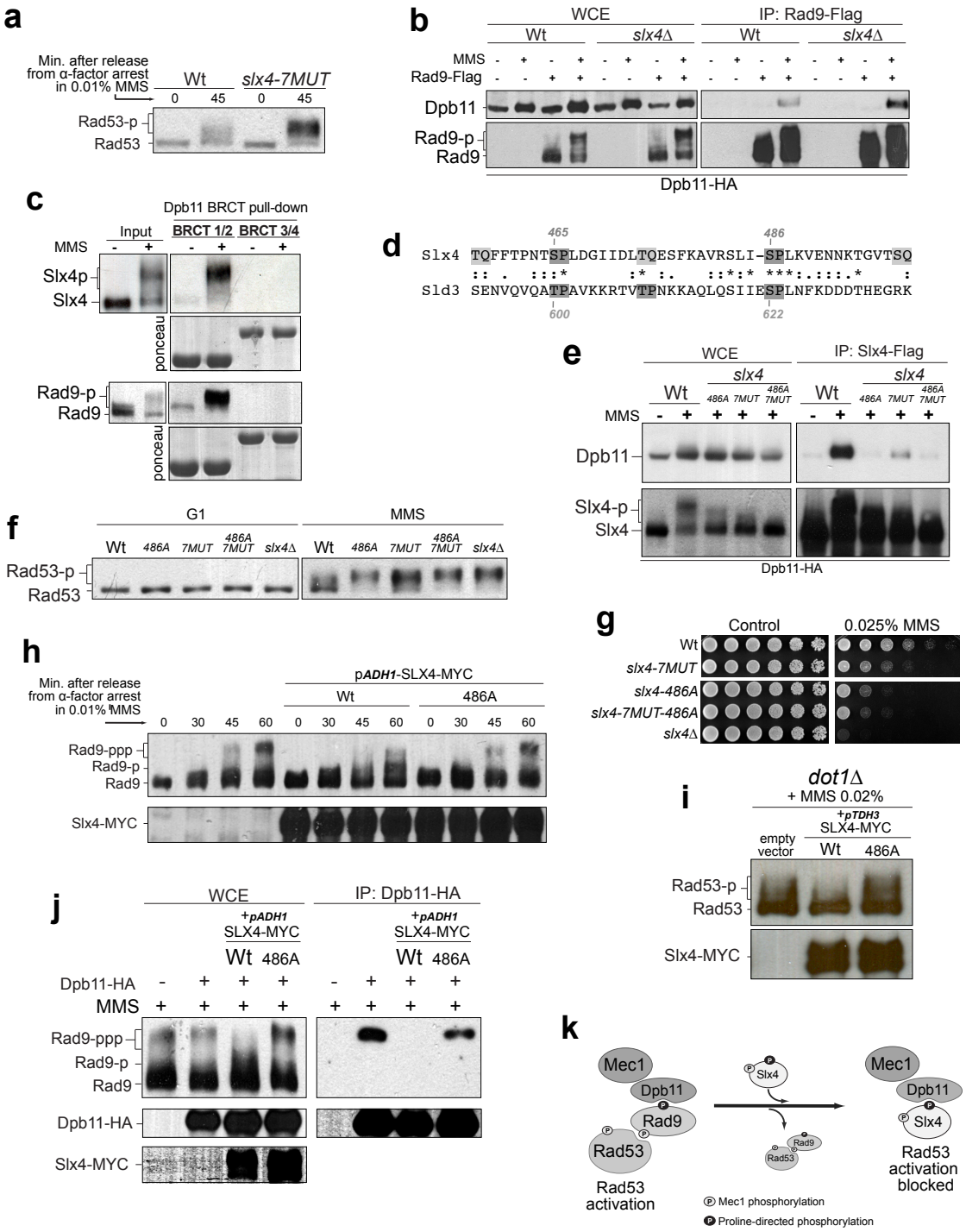

a
Min. after release from α-factor arrest in 0.01% MMS
Wt
slx4-7MUT
0 45 0 45
Rad53-p
Rad53
b
WCE
IP: Rad9-Flag
Wt
slx4Δ
Wt
slx4Δ
MMS
Rad9-Flag
Dpb11
Rad9-p
Rad9
Dpb11-HA
c
Dpb11 BRCT pull-down
Input
BRCT 1/2
BRCT 3/4
MMS
Slx4p
Slx4
ponceau
Rad9-p
Rad9
ponceau
d
465
486
Slx4 TQFFTPNTSPLDGIIDLTQESFKAVRSLI-SPLKVENNKTGVTSQ
:: . :::* :* :. ::*:* ***:.:::.* :
Sld3 SENVQVQATPAVKKRTVTFNKKAQLQSIIESPLNFKDDDTHEGRK
600
622
e
WCE
IP: Slx4-Flag
slx4
Wt
486A
7MUT
486A 7MUT
MMS
Dpb11
Slx4-p
Slx4
Dpb11-HA
f
G1
MMS
Wt
486A
7MUT
486A 7MUT
slx4Δ
Rad53-p
Rad53
g
Control
0.025% MMS
Wt
slx4-7MUT
slx4-486A
slx4-7MUT-486A
slx4Δ
h
Min. after release from α-factor arrest in 0.01% MMS
pADH1-SLX4-MYC
Wt
486A
0 30 45 60 0 30 45 60 0 30 45 60
Rad9-ppp
Rad9-p
Rad9
Slx4-MYC
i
dot1Δ
+ MMS 0.02%
+pTDH3
SLX4-MYC
empty vector
Wt
486A
Rad53-p
Rad53
Slx4-MYC
j
WCE
IP: Dpb11-HA
+pADH1
SLX4-MYC
Wt 486A
Wt 486A
Dpb11-HA
MMS
Rad9-ppp
Rad9-p
Rad9
Dpb11-HA
Slx4-MYC
k
Mec1
Dpb11
Rad9
Rad53
Slx4
Rad53 activation
Rad53 activation blocked
Mec1 phosphorylation
Proline-directed phosphorylation

Figure 3-2

Figure 3.2. **a**, Western blot showing the phosphorylation status of Rad53–HA in the indicated strains after MMS treatment. **b**, Co-immunoprecipitation (co-IP) of Dpb11 and Rad9 (see also Supplementary Figure 3.5); input levels and phosphorylation status of Rad9 are shown. **c**, Pull-down of Slx4–Flag or Rad9–HA from *S. cerevisiae* lysates using recombinant $BRCT^{1/2}$ and $BRCT^{3/4}$ from Dpb11; ponceau staining of the membrane is also shown. **d**, Interaction of the indicated Flag-tagged Slx4 mutants with Dpb11–HA; input levels and phosphorylation status of Slx4 are shown. **e**, Rad53 phosphorylation status in the indicated *slx4* mutants. The experiment was performed as described in Figure 3.1a. **f**, MMS sensitivity assay on the indicated *slx4* mutants. **g**, Rad9 phosphorylation status in cells expressing WT or mutant Slx4 from the endogenous *SLX4* promoter or the *ADH1* promoter. **h**, Rad53 phosphorylation status in *dot1*Δ cells expressing Slx4 from the endogenous *SLX4* promoter or the *TDH3* promoter. Cells were arrested in α-factor and released for 45 min in medium containing MMS. **i**, Co-IP of Dpb11 and Rad9. **j**, Model for the mechanism by which Slx4 counteracts Rad53 activation. G1, G1 cell-cycle phase; Rad9-p, hypophosphorylated Rad9; Rad9-ppp, hyperphosphorylated Rad9; WCE, whole cell extract.

Rtt107 Counteracts Rad9-dependent Rad53 Activation by Binding to Phosphorylated Histone H2a.

Slx4 forms a tight complex with the BRCT-domain-containing protein Rtt107, which is a DNA-repair scaffold that stabilizes the Slx4–Dpb11 interaction (Ohouo *et al.* 2010). Analysis of the phosphorylation status of Rad53 in *rtt107*Δ cells showed that DDC signalling is hyperactivated (Figure 3.3a), suggesting that Rtt107 has a similar function to Slx4 in counteracting Rad53 activation. Rtt107 recognizes $H2A^{pS129}$ through its carboxy-terminal pair of BRCT domains ($BRCT^{5/6}$), and this region is essential for the role of Rtt107 in the MMS response (Li *et al.* 2012) (Supplementary Figure 3.9 and 3.10). Furthermore, the S129A mutation (*h2a-S129A*) abrogated Slx4 phosphorylation on MMS treatment (Figure 3.3b), suggesting that the recruitment of Rtt107 to $H2A^{pS129}$ occurs upstream of Slx4–Dpb11 complex formation. Because $H2A^{pS129}$ can function as a positive regulator of Rad9-dependent Rad53 activation (Javaheri *et al.* 2006), I reasoned that the hyperactivation of Rad53 in *rtt107*Δ cells could be suppressed by *h2a-S129A*. *rtt107*Δ cells expressing the $H2A^{(S129A)}$ mutant had lower Rad53 activation and MMS sensitivity than did *rtt107*Δ cells expressing wild-type H2A (Figure 3.3c, d). Disruption of the Rad9–H2A interaction by mutation of the C-terminal pair of BRCT domains of Rad9 (K1088M) partially rescued the MMS sensitivity of *rtt107*Δ cells (Supplementary Figure 3. 11a). A similar rescue was also observed when overexpressing $BRCT^{5/6}$ of Rtt107 (Supplementary Figure 3.11b). Taken together, these results show that, like Slx4, Rtt107 also counteracts DDC signalling. The anti-checkpoint function of Rtt107 depends on its recognition of $H2A^{pS129}$, a step that is required subsequently for the

assembly of the Slx4–Dpb11 complex. Thus, the Slx4–Rtt107 complex functions as a negative regulator of activation of the kinase Rad53 by physically interacting with two positive regulators of the adaptor Rad9: Dpb11 and H2A^{pS129}.

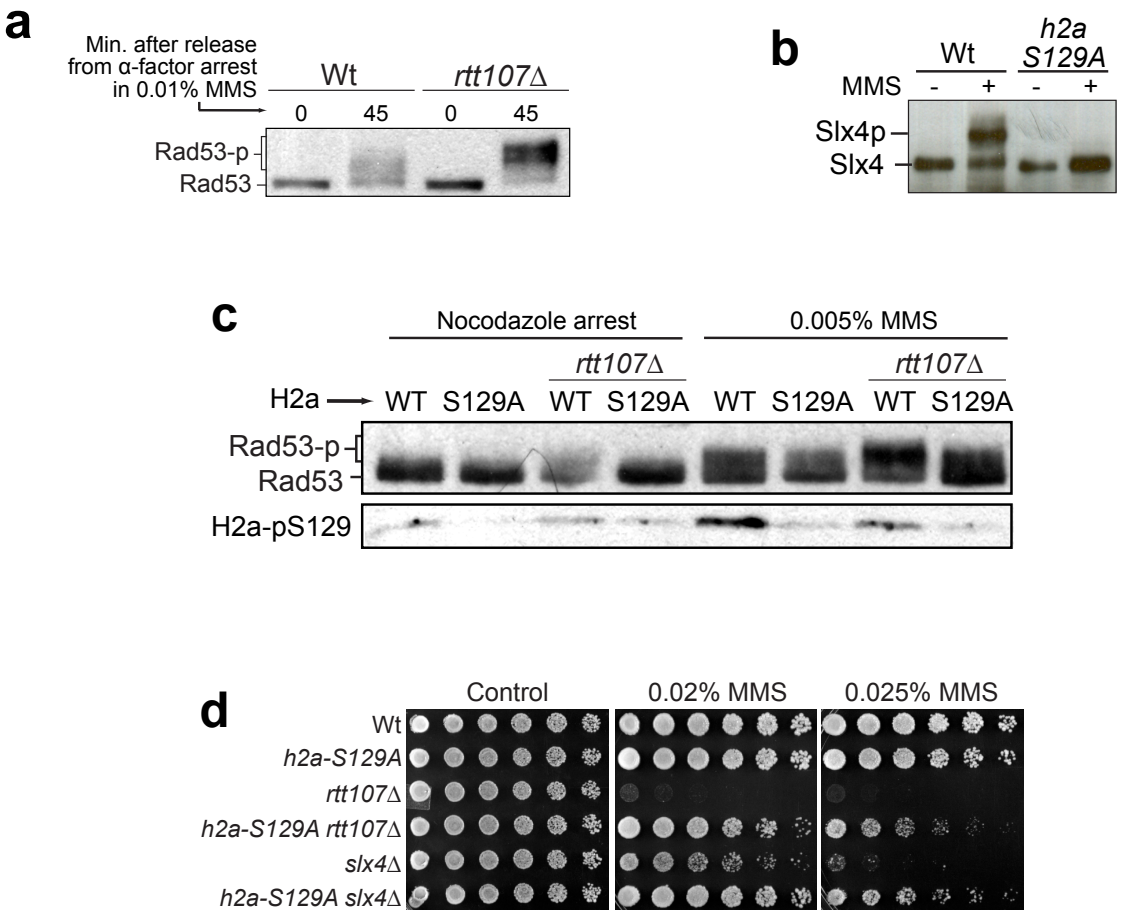

a
Min. after release from α-factor arrest in 0.01% MMS
Wt
rtt107Δ
0
45
0
45
Rad53-p
Rad53
b
Wt
h2a S129A
MMS
-
+
-
+
Slx4p
Slx4
c
Nocodazole arrest
0.005% MMS
rtt107Δ
rtt107Δ
H2a
WT
S129A
WT
S129A
WT
S129A
WT
S129A
Rad53-p
Rad53
H2a-pS129
d
Control
0.02% MMS
0.025% MMS
Wt
h2a-S129A
rtt107Δ
h2a-S129A rtt107Δ
slx4Δ
h2a-S129A slx4Δ

Figure 3-3

Figure 3.3 **a**, Western blot showing the phosphorylation status of Rad53–HA in the indicated strains after MMS treatment. **b**, Western blot showing Slx4–Flag phosphorylation status. **c**, Western blot showing phosphorylation status of Rad53 and $H2A^{pS129}$. Indicated strains expressing HA-tagged Rad53 were arrested in nocodazole and released for 60 min in medium containing MMS. **d**, MMS sensitivity assay on the indicated mutants.

Slx4 Sensitizes *mrc1Δ* Cells to HU-induced Replication Stress.

To further test the anti-checkpoint function of the Slx4–Rtt107 complex, I developed an alternative experimental set-up in which the presence of Slx4 would sensitize cells to replication stress by decreasing DDC signalling below the level required for a proper cellular response. For this set-up, I used cells lacking Mrc1, which is a checkpoint adaptor that works in parallel with Rad9. Mrc1 mediates Rad53 activation at stalled replication forks and therefore has a more central role than Rad9 in Rad53 activation in response to hydroxyurea (Alcasabas *et al.* 2001). On hydroxyurea treatment, *mrc1*Δ cells rely solely on Rad9 to activate Rad53. The interaction of Slx4 with Dpb11 was enhanced in *mrc1*Δ cells (Figure 3.4a), especially in response to hydroxyurea, suggesting that the Slx4–Rtt107 complex actively counteracts Rad9 in *mrc1*Δ cells (Figure 3.4b). Consistent with our finding that the Slx4–Dpb11 interaction requires $H2A^{pS129}$, high levels of $H2A^{pS129}$ accumulated close to the origins of replication in *mrc1*Δ cells after hydroxyurea treatment (Figure 3.4c). Because I expect that the kinase activity of Rad53 is limiting in *mrc1*Δ cells, I reasoned that deletion of *SLX4* would be beneficial in hydroxyurea-treated *mrc1*Δ cells, allowing more activation of Rad53 through Rad9. Consistent with this idea, *mrc1*Δ*slx4*Δ cells had a significantly higher hydroxyurea resistance than *mrc1*Δ cells (Figure 3.4d), and this resistance correlated with higher Rad53 activation (Figure 3.4e).

Taken together, these results show that Slx4 sensitizes *mrc1*Δ cells to replication stress, and they provide strong additional evidence that the Slx4–Rtt107 complex counteracts Rad9 in response to replication-induced lesions (see detailed

model in Supplementary Figure 3.12). Because this function of the Slx4–Rtt107 complex depends on Mec1-dependent phosphorylation, I propose that Slx4–Rtt107 is involved in a mechanism that I have named DAMP — dampens checkpoint adaptor-mediated phospho-signalling — by which DDC signalling self-monitors its activation state. I speculate that by uncoupling upstream Mec1 signalling from downstream Rad53 activation, DAMP could allow Mec1 to maintain control over specific effectors, such as repair enzymes, without an aberrant arrest in cell-cycle progression.

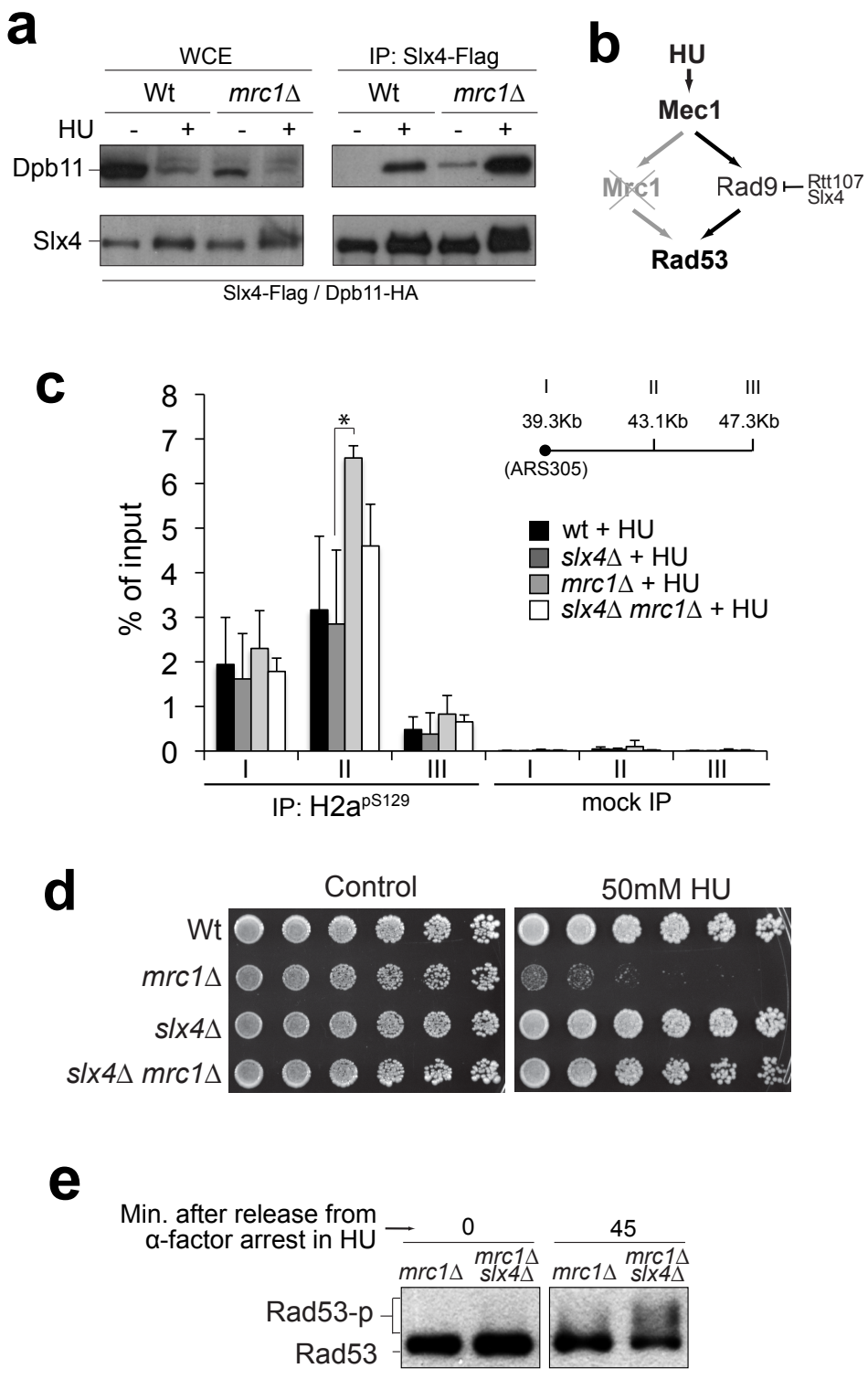
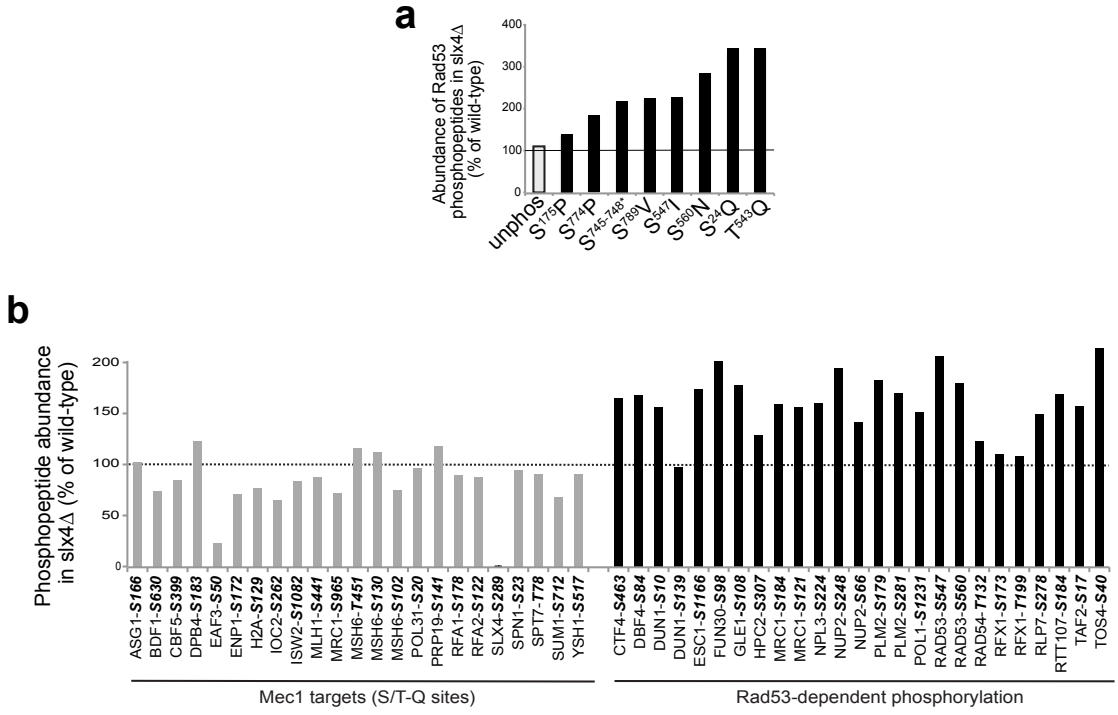

a
WCE
IP: Slx4-Flag
Wt
mrc1Δ
Wt
mrc1Δ
HU - + - + - + - +
Dpb11
Slx4
Slx4-Flag / Dpb11-HA
b
HU
Mec1
Mrc1
Rad9
Rtt107
Slx4
Rad53
c
% of input
8
7
6
5
4
3
2
1
0
I
II
III
39.3Kb
43.1Kb
47.3Kb
(ARS305)
wt + HU
slx4Δ + HU
mrc1Δ + HU
slx4Δ mrc1Δ + HU
IP: H2apS129
mock IP
d
Control
50mM HU
Wt
mrc1Δ
slx4Δ
slx4Δ mrc1Δ
e
Min. after release from α-factor arrest in HU
0
45
mrc1Δ
mrc1Δ slx4Δ
mrc1Δ
mrc1Δ slx4Δ
Rad53-p
Rad53

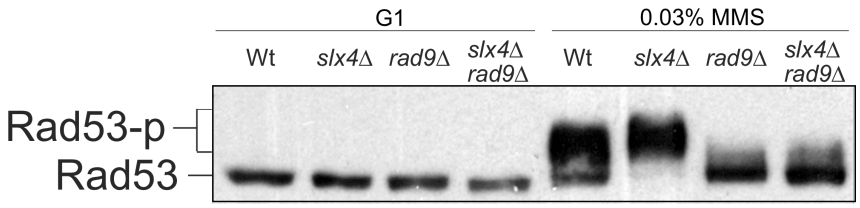
Figure 3-4

Figure 3.4 **a**, Co-IP of Dpb11 and Slx4 after 2 h treatment with 0.1 M hydroxyurea (HU). **b**, Model for the action of Slx4 and Rtt107 in *mrc1*Δ cells. **c**, HU treatment of *mrc1*Δ cells leads to H2A^{pS129} accumulation near an origin of replication. Chromatin immunoprecipitation (ChIP) analysis of H2A^{pS129} at neighbouring *ARS305* regions I, II and III in the indicated strains after exposure to 0.2 M HU. Data are presented as mean + s.e.m. (n = 3). *, significant difference (P = 0.025), as analysed by unpaired, two-tailed student's t-test. **d**, HU sensitivity assay on the indicated mutants. **e**, Western blot showing the phosphorylation status of Rad53–HA after treatment with 10 mM HU. kb, kilobases.



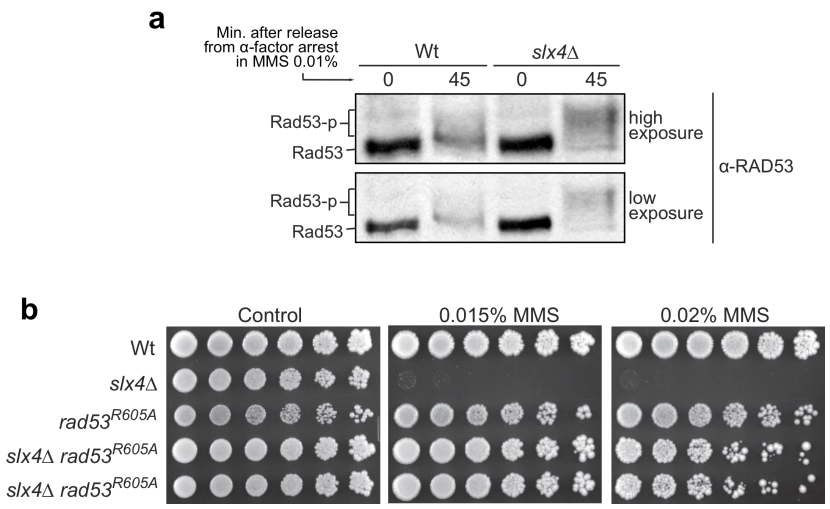
Supplementary figure 3-1

Supplementary Figure 3.1. Rad53, but not Mec1, is hyperactivated in *slx4Δ* cells. a, Quantitative mass spectrometry analysis of the relative abundance of Rad53 phosphopeptides containing the indicated phosphorylation sites, in wild-type and *slx4Δ* cells grown in 0.01% MMS for 1 h. Rad53-HA was immuno-precipitated, trypsinized and phosphorylated peptides were purified via IMAC resin prior to mass spectrometry analysis. The average percentage for unphosphorylated Rad53 peptides ("unphos") is indicated as a control for the ratio of total Rad53 protein abundance. Asterisk indicates that a phosphorylation was detected within the indicated residues (see Supplementary Table 3.3 for more details). b, Quantitative phosphoproteome analysis of wild-type and *slx4Δ* cells (both with untagged Rad53) grown as in a. Over 3570 phosphopeptides were identified/quantified and the results for phosphopeptides containing a phosphorylation at the indicated sites, known to be Mec1 targets or Rad53-dependent phosphorylation, are shown (see Supplementary Table 3.4 and Methods for details).



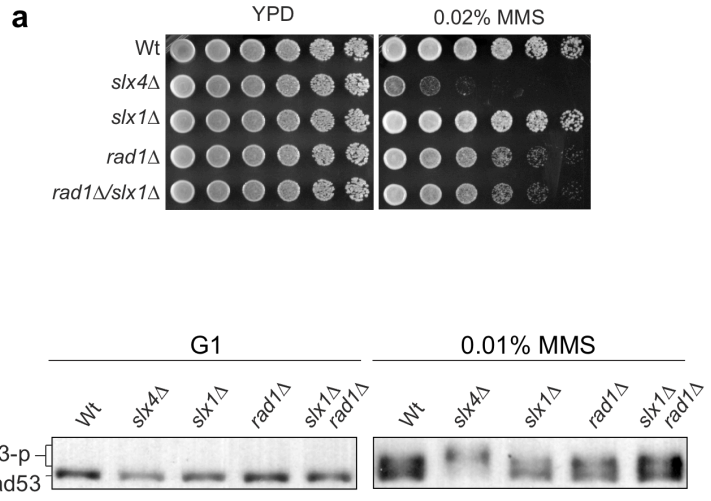
Supplementary figure 3-2

Supplementary Figure 3.2. Rad53 activation in *slx4Δ* cells is Rad9-dependent. Western blot showing the phosphorylation status of Rad53-HA in indicated strains. Cells were arrested in G1 with alpha-factor and released for 60 minutes in media containing 0.03 % MMS.



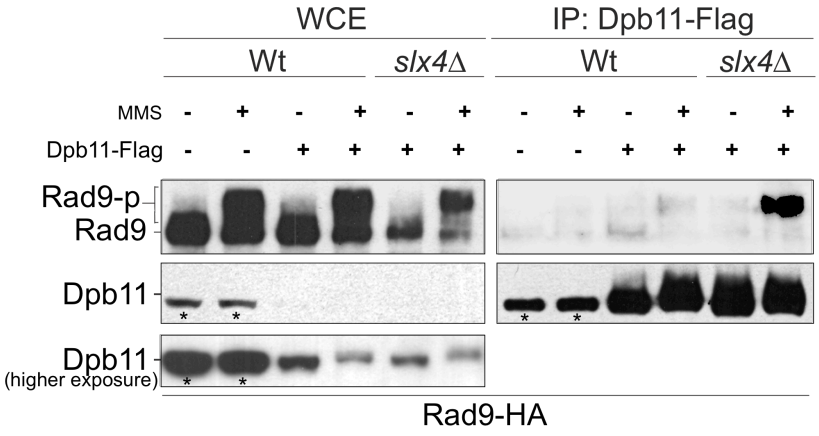
Supplementary figure 3-3

Supplementary Figure 3.3. Rad53 hyperactivation and FHA2 mutation-dependent phenotypic rescue in *slx4Δ* cells carrying untagged RAD53 alleles. a, Cells lacking SLX4 and carrying an untagged allele of RAD53 also exhibit MMS-induced hyperactivation of Rad53. Rad53 was detected using anti-Rad53 antibody from Santa Cruz (yc-19, cat. # sc-6749). b, The MMS-sensitivity of cells lacking SLX4 and carrying an untagged allele of RAD53 can also be rescued by a disrupting mutation in the FHA2 domain of Rad53 (R605A). All of the indicated strains were derived from a freshly sporulated diploid. Last two rows represent distinct spores.



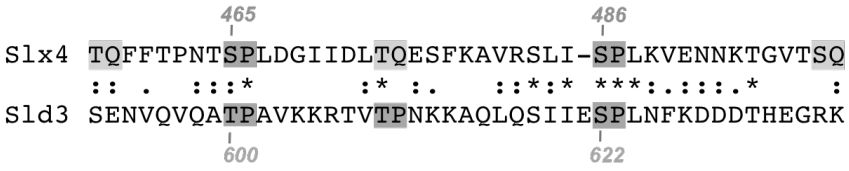
Supplementary figure 3-4

Supplementary Figure 3.4. Slx4 has an Slx1 and Rad1-independent function. a, MMS sensitivity assay of the indicated strains. b, Western blot for Rad53 phospho-status. Cells were released from G1 arrest and grown for 45 minutes in media containing 0.01% MMS.



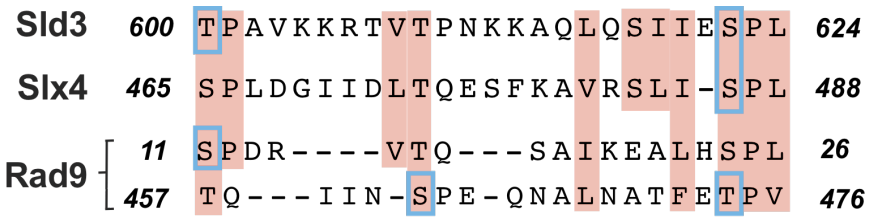
Supplementary figure 3-5

Supplementary Figure 3.5. Co-immunoprecipitation between Dpb11 and Rad9 in wild-type and *slx4Δ* cells. Asterisk indicates a contaminating band that is specifically present in Dpb11 untagged cells.



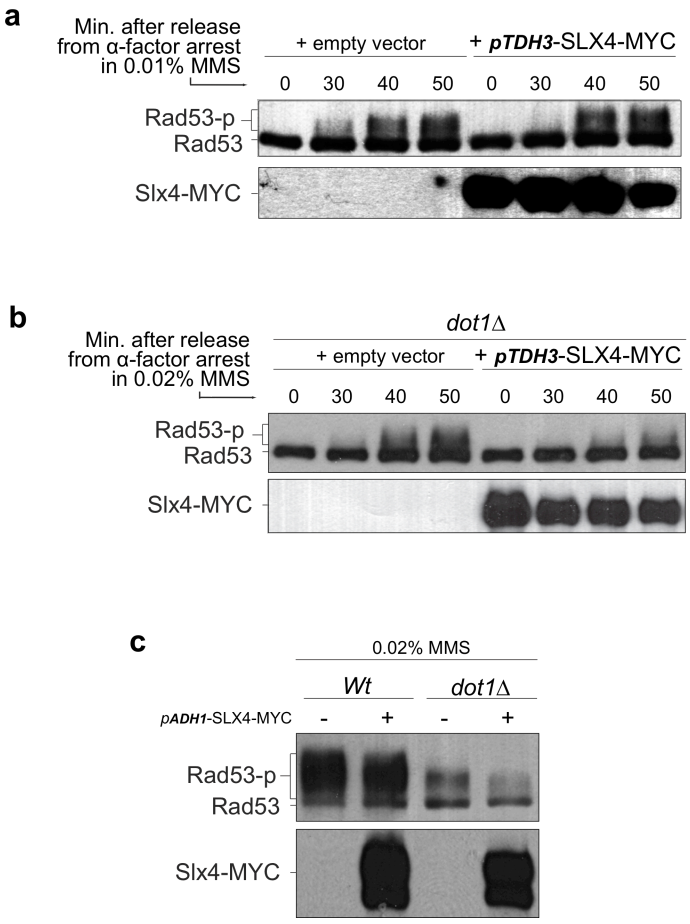
Supplementary figure 3-6

Supplementary Figure 3.6. Manual alignment of the Dpb11-binding region of Sld3 with amino acids 457-500 of Slx4. Proline-directed and Mec1 consensus phosphorylation sites are indicated in dark and light boxes, respectively. Numbers indicate the amino acid position in protein sequence. Serines 600 and 622 of Sld3 were previously shown to be important for Dpb11-Sld3 interaction (Tanaka *et al.* 2007, Zegerman and Diffley 2007).



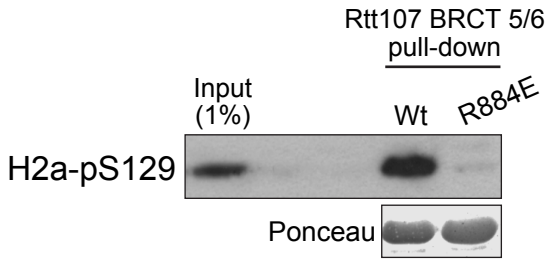
Supplementary figure 3-7

Supplementary Figure 3.7. Manual alignment of proline-directed sites in Slx4, Rad9 and Sld3 important for binding to Dpb11. Blue boxes indicate the residues that have been shown to be important for Dpb11 interaction with Sld3 (Tanaka *et al.* 2007, Zegerman and Diffley 2007), Rad9 (Granata *et al.* 2010, Pfander and Diffley 2011) and Slx4 (this study). Red shade indicates sequence similarities.



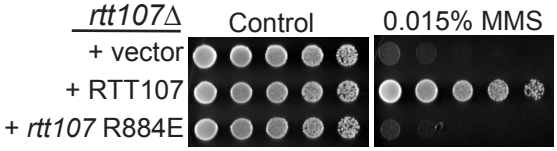
Supplementary figure 3-8

Supplementary Figure 3.8. Overexpression of Slx4 reduces Rad53 activation. Rad53 phospho-status in (a) wild-type and (b) *dot1Δ* cells expressing Slx4 from its endogenous promoter or overexpressing Slx4 from a plasmid under the control of a TDH3 promoter. (c) Rad53 phospho-status in wild-type and *dot1Δ* cells expressing Slx4 from its endogenous promoter or overexpressing Slx4 from an ADH1 promoter integrated in the genome, which replaced the endogenous SLX4 promoter. The experiment in (c) was performed in YPD media and cells were collected 50 minutes after release from alpha-factor arrest.



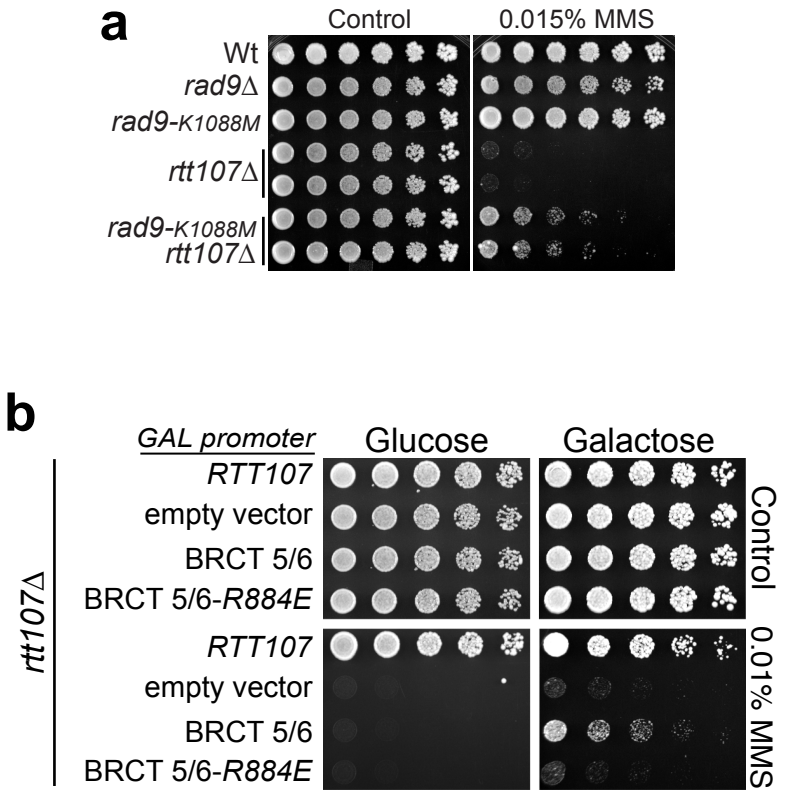
Supplementary figure 3-9

Supplementary Figure 3.9. Rtt107 binds to H2A^{pS129} via its C-terminal pair of BRCT domains. Recombinant BRCT5/6 (amino acids 750-1070) of Rtt107 carrying an N-terminal PATH tag (Protein A, TEV site and 6 x His), and pre-bound to IgG beads, was used to pull-down histone H2A phosphorylated at serine 129 from MMS-treated yeast lysates. The corresponding R884E mutation in the S. pombe ortholog Brc1 was shown to abolish its interaction with phosphorylated H2A (William *et al.* 2010).



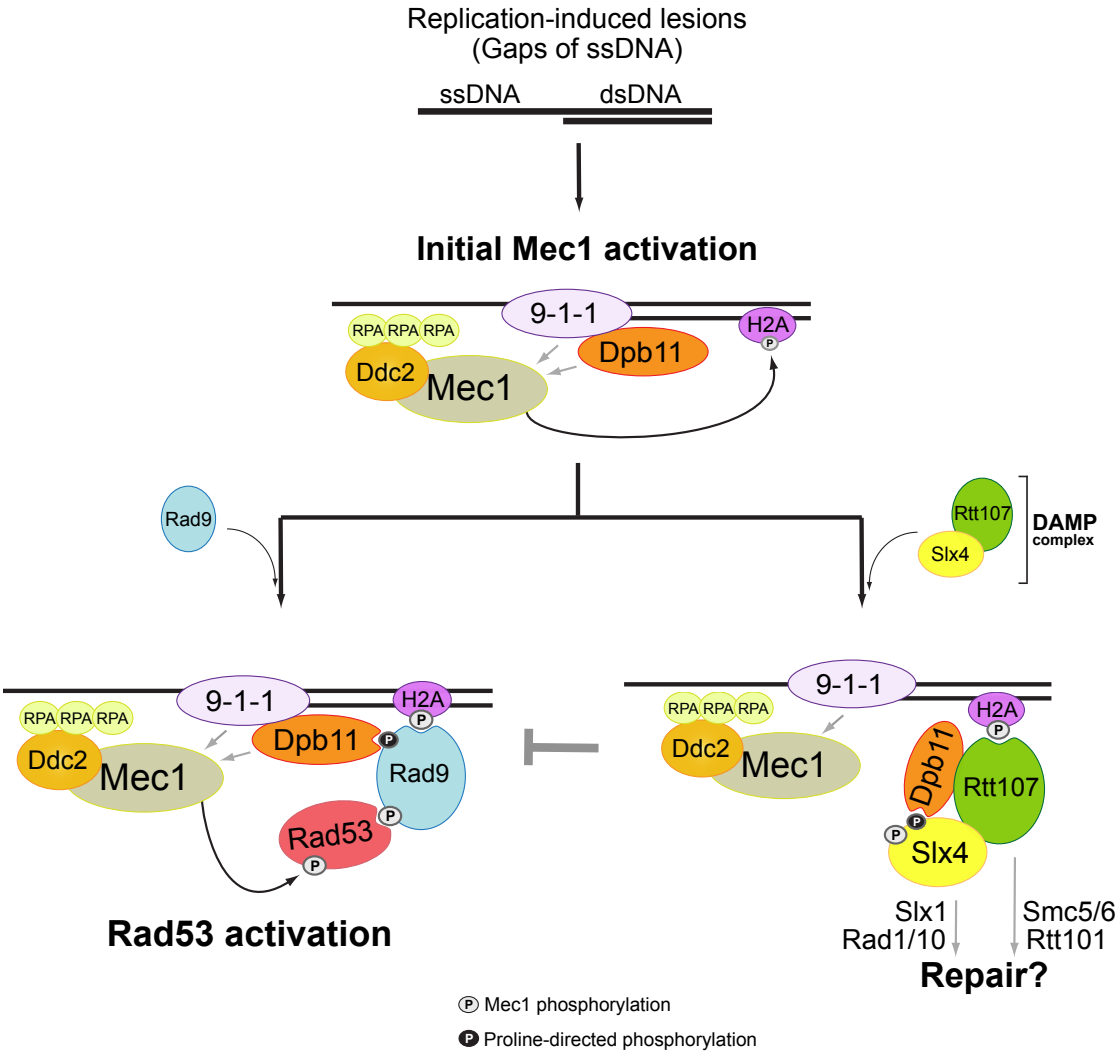
Supplementary figure 3-10

Supplementary Figure 3.10. The C-terminal pair of BRCT domains of Rtt107 is crucial for the response to MMS. MMS sensitivity assay of *rtt107Δ* cells expressing indicated forms of Rtt107 from a pRS416 vector.



Supplementary figure 3-11

Supplementary Figure 3.11. Sensitivity of *rtt107Δ* cells to MMS is rescued by disrupting the Rtt107-H2A interaction. a, MMS sensitivity assay. K1088M mutation in Rad9 has been previously shown to impair the ability of Rad9 to interact with phospho-H2A (Hammet *et al.* 2007). b, MMS sensitivity assay for *rtt107Δ* cells overexpressing the indicated constructs under the control of a galactose-inducible promoter in a pYES vector.



Supplementary figure 3-12

Supplementary Figure 3.12. Detailed speculative model for modulation of DDC signaling by the Slx4-Rtt107 complex. Initial Mec1 activation: Replication-induced lesions, such as gaps of ssDNA left behind a moving replication fork, recruit the Mec1-Ddc2 complex via RPA-bound ssDNA. The 9-1-1 complex is recruited to ssDNA-dsDNA junctions and then recruits Dpb11 to cooperate in the activation of Mec1. Activated Mec1 phosphorylates H2A at regions of dsDNA nearby lesions. Rad9-dependent Rad53 activation: Phospho-H2A and Dpb11 contribute to stabilize Rad9 at lesions. Rad9 is then phosphorylated by Mec1 to create a docking site for the recruitment of Rad53. Rad9-bound Rad53 undergoes kinase activation by Mec1 phosphorylation and subsequent Rad53 auto-phosphorylation. Modulation of Rad53 activation by a DAMP mechanism: The Slx4-Rtt107 complex antagonizes Rad9-dependent Rad53 activation by physically interacting with phospho-H2A and Dpb11, and therefore competing for the same binding sites important for Rad9 stabilization at lesions. I propose that the engagement of the Slx4-Rtt107 complex at sites of lesions is established by Mec1-dependent and proline-directed phosphorylation events, and functions to tune the levels of Rad53 activation. It is still unclear if Slx4 and Rtt107 rely on the physical interactions with H2A and Dpb11 to coordinate the action of structure-specific endonucleases (Slx1 and Rad1/Rad10) and other repair factors at sites of lesion.

Phospho-peptides	geometric mean (Wt/slx4Δ)	Geometric standard deviation	Abundance in slx4Δ (% of Wt)	Phospho site
K.IPAHAPIRYTphosQPK.S	0.3	1	333.33	543
R.FLIEKFSphosQEQIGENIVCR.V	0.3	1.21	333.33	24
K.LLHSphosNNTENVK.S	0.37	1.05	270.27	560
K.SphosIEAETR.E	0.46	1.03	217.39	547
R.IHSphosVSLSQSQIDPSK.K	0.46	1.03	217.39	789
R.ANQPSA(SSSS)phosMSAK.K	0.51	1.05	196.08	745-748
K.KPPVSDTNNNGNNSVLNDLVESphosPINANTGNILK.R	0.6	1.24	166.67	774
K.IASphosPGLTSSTASSMVANK.T	0.76	1.06	131.58	175
Unphosphorylated peptides				
R.KLQMEQQLQEQDQEDQDGK.I	0.8	1.1	125	
R.GKDTSVSPDEYEER.N	0.89	1.18	112.36	
K.FLLQDGDEIK.I	0.96	1.04	104.17	
K.TFCGTLAYVAPEVIR.G	0.97	1.02	103.09	
K.DTSVSPDEYEER.N	0.99	1.06	101.01	
K.LQMEQQLQEQDQEDQDGKIQGFK.I	1	1	100	
K.LLENMDDAQYEFVK.A	1.01	1	99.01	
R.KLQMEQQLQEQDQEDQDGKIQGFK.I	1.04	1	96.15	

Supplementary table 3-3

Supplementary Table 3.3. Quantitative data of Rad53 peptides identified in the experiment shown in Supplementary Figure 3.1a. Wild-type and *slx4Δ* cells containing HA-tagged Rad53 were grown in heavy and light media, respectively. All unphosphorylated Rad53 peptides still detected after the IMAC purification step are also listed and were used as a measure of the difference of Rad53 protein levels in wild-type and *slx4Δ* cells.

Mec1-dependent Rad53-independent phosphorylation	geometric mean (slx4Δ/Wt)	Geometric standard deviation	Abundance in slx4Δ (% of Wt)	Phospho site	orf	gene
K.RDSphosQSFLDDTVK.E	1.02	1	102	166	YIL130W	ASG1
K.ALSphosQEEQSR.Q	0.74	1	74	630	YLR399C	BDF1
K.EYVPLDNAEQSTSSSphosQETK.E	0.85	1	85	399	YLR175W	CBF5
K.TESphosQDVETR.V	1.23	1	123	183	YDR121W	DPB4
K.MYTSIPNDKPGGSSphosQATK.E	0.23	1.52	23	50	YPR023C	EAF3
R.EKESphosQVEDMQDDEPLANEQNTSR.G	0.71	1	71	172	YBR247C	ENP1
K.ATKASphosQEL.-	0.77	1	77	129	YDR225W	HTA1
K.EGSSSphosQVSQTLNIPIK.L	0.65	1.01	65	262	YLR095C	IOC2
R.EDTPLSphosQNESTR.A	0.84	1.07	84	1082	YOR304W	ISW2
K.VTNVSHSphosQEAEKLTLNESEQPR.D	0.88	1	88	441	YMR167W	MLH1
K.SFTNSphosQTDSTTSK.T	0.72	1.03	72	965	YCL061C	MRC1
R.EEPGNFYNETphosQLDSSTIVQK.L	1.16	1.06	116	451	YDR097C	MSH6
K.STTTDEDLSSSphosQSR.R	1.12	1.11	112	130	YDR097C	MSH6
R.SDIMHSphosQEPQSDTMLNSNTTEPK.S	0.75	1	75	102	YDR097C	MSH6
R.SLQDENLSphosQPR.T	0.97	1	97	20	YJR006W	POL31
K.SSphosQQAVAITR.E	1.18	1.06	118	141	YLL036C	PRP19
K.FANENPNSphosQK.T	0.9	1.07	90	178	YAR007C	RFA1
K.GYGSphosQVAQQFEIGGYVK.V	0.88	1.27	88	122	YNL312W	RFA2
K.SSNSLDNQESSphosQQR.L	0	1	0	289	YLR135W	SLX4
K.VVEATPEDGTASSphosQK.S	0.95	1.01	95	23	YPR133C	SPN1
K.SFPLTphosQEEEHHGAVSPAVDTR.S	0.91	1	91	78	YBR081C	SPT7
R.KTPGDEETTTFVPLENSphosQPSDTIR.K	0.68	1.23	68	712	YDR310C	SUM1
K.IEPIKEENEDNLDSphosQAEK.G	0.91	1.03	91	517	YLR277C	YSH1
Rad53-dependent phosphorylation						
K.THSphosFPISLANTGK.F	1.65	1.07	165	463	YPR135W	CTF4
R.ARSphosIEGAVQVSK.G	1.68	1.1	168	84	YDR052C	DBF4
K.REHSphosGDVTDSSFK.R	1.56	1	156	10	YDL101C	DUN1
K.YASSSSphosTDIENDDEKVSSESR.S	0.98	1.22	98	142	YDL101C	DUN1
K.VVDVAESphosFVK.K	1.74	1	174	1166	YMR219W	ESC1
K.SNSphosFNLQSAR.E	2.01	1	201	98	YAL019W	FUN30
R.ASHSphosSLLDNAK.N	1.78	1	178	108	YDL207W	GLE1
R.SISphosSVTNSDSNISSSK.K	1.29	1.02	129	307	YBR215W	HPC2
R.IDSphosSGATSQTQPIK.S	1.59	1.01	159	184	YCL061C	MRC1
K.NVSSSphosFTQTQR.I	1.56	1.1	156	121	YCL061C	MRC1
R.ENSphosLETTFSSVNTR.D	1.6	1.06	160	224	YDR432W	NPL3
K.KTETNAKPFSphosFSSATSTTEQTK.S	1.95	1	195	248	YLR335W	NUP2
K.QASSphosFSFLNR.A	1.42	1.09	142	66	YLR335W	NUP2
K.LVAEEPLTSphosQNTK.Q	1.83	1	183	179	YDR501W	PLM2
K.FVPVEHSphosPR.T	1.7	1.11	170	281	YDR501W	PLM2
R.IDSphosFNVVR.L	1.51	1.08	151	1231	YNL102W	POL1
K.SphosIEAETR.E	2.06	1	206	547	YPL153C	RAD53
K.LLHSphosNNTENVK.S	1.8	1	180	560	YPL153C	RAD53
R.SFTphosVPIK.G	1.23	1	123	132	YGL163C	RAD54
R.SNSphosSAYPLSFK.S	1.1	1.1	110	173	YLR176C	RFX1
R.TNTphosFPSIPSSTKK.Q	1.08	1	108	199	YLR176C	RFX1
R.EVSphosGFGSLNR.L	1.49	1.04	149	278	YNL002C	RLP7
K.LTFGSFSphosSSSTNK.H	1.69	1	169	186	YHR154W	RTT107
R.AIVSESphosSTLHEMK.F	1.57	1	157	17	YCR042C	TAF2
K.TASphosESIGNFGK.G	2.14	1	214	40	YLR183C	TOS4

Supplementary table 3-4

Supplementary Table 3.4. Quantitative data for phosphopeptides identified in the phosphoproteome experiment shown in Supplementary Fig. 3.1b. Wild-type and *slx4Δ* cells were grown in light and heavy media, respectively. From over 3570 phosphopeptides identified in the experiment, the table shows data for the phosphopeptides that contain phosphorylation dependent on Mec1 and/or Rad53 as defined previously (smolka *et al.* 2007, Chen *et al.* 2010, Personal communication at the Eukaryotic DNA Replication & Genome Maintenance meeting. Cold Spring Harbor, NY, 2009).

REFERENCES

Weinert, T. A. & Hartwell, L. H. The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science 241, 317–322 (1988).

Clerici, M. *et al.* Hyperactivation of the yeast DNA damage checkpoint by *TEL1* and *DDC2* overexpression. EMBO J. 20, 6485–6498 (2001).

Pellicioli, A., Lee, S. E., Lucca, C., Foiani, M. & Haber, J. E. Regulation of *Saccharomyces* Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. Mol. Cell 7, 293–300 (2001).

Fekairi, S. *et al.* Human SLX4 is a Holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases. Cell 138, 78–89 (2009).

Svendsen, J. M. *et al.* Mammalian BTBD12/SLX4 assembles a Holliday junction resolvase and is required for DNA repair. Cell 138, 63–77 (2009).

Muñoz, I. M. *et al.* Coordination of structure-specific nucleases by human SLX4/BTBD12 is required for DNA repair. Mol. Cell 35, 116–127 (2009).

Fricke, W. M. & Brill, S. J. Slx1–Slx4 is a second structure-specific endonuclease functionally redundant with Sgs1–Top3. Genes Dev. 17, 1768–1778 (2003).

Stoepker, C. *et al.* SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. Nature Genet. 43, 138–141 (2011).

Kim, Y. *et al.* Mutations of the *SLX4* gene in Fanconi anemia. Nature Genet. 43, 142–146 (2011).

Roberts, T. M. *et al.* Slx4 regulates DNA damage checkpoint-dependent phosphorylation of the BRCT domain protein Rtt107/Esc4. Mol. Biol. Cell 17, 539–548 (2006).

Schwartz, M. F. *et al.* Rad9 phosphorylation sites couple Rad53 to the *Saccharomyces cerevisiae* DNA damage checkpoint. Mol. Cell 9, 1055–1065 (2002).

Schwartz, M. F., Lee, S. J., Duong, J. K., Eminaga, S. & Stern, D. F. FHA domain-mediated DNA checkpoint regulation of Rad53. Cell Cycle 2, 381–394 (2003).

Tercero, J. A., Longhese, M. P. & Diffley, J. F. A central role for DNA replication forks in checkpoint activation and response. Mol. Cell 11, 1323–1336 (2003).

Ohouo, P. Y., Bastos de Oliveira, F. M., Almeida, B. S. & Smolka, M. B. DNA damage signaling recruits the Rtt107–Slx4 scaffolds via Dpb11 to mediate replication stress response. Mol. Cell 39, 300–306 (2010).

Navadgi-Patil, V. M. & Burgers, P. M. Yeast DNA replication protein Dpb11 activates the Mec1/ATR checkpoint kinase. J. Biol. Chem. 283, 35853–35859 (2008).

Mordes, D. A., Nam, E. A. & Cortez, D. Dpb11 activates the Mec1–Ddc2 complex. Proc. Natl Acad. Sci. USA 105, 18730–18734 (2008).

Granata, M. *et al.* Dynamics of Rad9 chromatin binding and checkpoint function are mediated by its dimerization and are cell cycle-regulated by CDK1 activity. PLoS Genet. 6, e1001047 (2010).

Pfander, B. & Diffley, J. F. Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment. EMBO J. 30, 4897–4907 (2011).

Tanaka, S. *et al.* CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. Nature 445, 328–332 (2007).

Zegerman, P. & Diffley, J. F. Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. Nature 445, 281–285 (2007).

Puddu, F. *et al.* Phosphorylation of the budding yeast 9-1-1 complex is required for Dpb11 function in the full activation of the UV-induced DNA damage checkpoint. Mol. Cell. Biol. 28, 4782–4793 (2008).

Li, X. *et al.* Structure of C-terminal tandem BRCT repeats of Rtt107 protein reveals critical role in interaction with phosphorylated histone H2A during DNA damage repair. J. Biol. Chem. 287, 9137–9146 (2012).

Javaheri, A. *et al.* Yeast G1 DNA damage checkpoint regulation by H2A phosphorylation is independent of chromatin remodeling. Proc. Natl Acad. Sci. USA 103, 13771–13776 (2006).

Alcasabas, A. A. *et al.* Mrc1 transduces signals of DNA replication stress to activate Rad53. Nature Cell Biol. 3, 958–965 (2001).

Smolka, M. B. *et al.* An FHA domain-mediated protein interaction network of Rad53 reveals its role in polarized cell growth. J. Cell Biol. 175, 743–753 (2006).

Albuquerque, C. P. *et al.* A multidimensional chromatography technology for in-depth phosphoproteome analysis. Mol. Cell. Proteomics 7, 1389–1396 (2008).

Smolka, M. B., Albuquerque, C. P., Chen, S. H. & Zhou, H. Proteome-wide identification of *in vivo* targets of DNA damage checkpoint kinases. Proc. Natl Acad. Sci. USA 104, 10364–10369 (2007).

Smolka, M. B. *et al.* Dynamic changes in protein–protein interaction and protein phosphorylation probed with amine-reactive isotope tag. Mol. Cell. Proteomics 4, 1358–1369 (2005).

Chen, S. H., Albuquerque, C. P., Liang, J., Suhandynata, R. T. & Zhou, H. A proteome-wide analysis of kinase-substrate network in the DNA damage response. J. Biol. Chem. 285, 12803–12812 (2010).

Petesch, S. J. & Lis, J. T. Rapid, transcription-independent loss of nucleosomes over a large chromatin domain at *Hsp70* loci. Cell 134, 74–84 (2008).

Williams, J. S. et al. gammaH2A binds Brc1 to maintain genome integrity during S-phase. EMBO J 29, 1136-1148, doi:emboj2009413 [pii] 10.1038/emboj.2009.413 (2010).

Hammet, A., Magill, C., Heierhorst, J. & Jackson, S. P. Rad9 BRCT domain interaction with phosphorylated H2AX regulates the G1 checkpoint in budding yeast. EMBO Rep 8, 851-857, doi:7401036 [pii] 10.1038/sj.embor.7401036 (2007).

Szilard, R. K. et al. Systematic identification of fragile sites via genome-wide location analysis of gamma-H2AX. Nat Struct Mol Biol 17, 299-305, doi:10.1038/nsmb.1754 (2010).

Chapter 4

Conclusions and future directions

CONCLUSIONS

Replication checkpoint signaling and DNA repair are critical for the preservation of genomic integrity. Here I demonstrate that the Rtt107-Slx4 complex plays crucial roles in the coordination of checkpoint signaling and DNA repair. My initial work (Ohouo et al, 2010) revealed that Mec1 helps recruit the Rtt107-Slx4 complex to the checkpoint activator protein Dpb11. More recently, I found that the specific disruption of this recruitment leads to aberrant Rad53 activation and hypersensitivity to DNA alkylation induced replication stress (Ohouo et al, 2013). Two potential models arise from these findings: (1) The recruitment of the Rtt107-Slx4 complex to Dpb11 functions to coordinate the action of specific repair factors at sites of lesions; (2) The recruitment of the Rtt107-Slx4 complex to Dpb11 directly modulates the activation of Rad53. These models are not necessarily mutually exclusive. It is conceivable that the Rad53 modulation and repair functions of the Rtt107-Slx4 complex act synergistically to promote efficient repair. In fact, I favor such a comprehensive model because of the following reasons: 1) the rescue of the MMS sensitivity of *slx4Δ* cells by the *rad53 R605A* hypomorphic allele is not complete. 2) Cells lacking *RTT107* or *SLX4*, display persistent and aberrant DNA structures along with excessive Rad53 activation, (Flott et al., 2007). 3) Our analysis of Dpb11 pull downs reveals that Slx1 is an integral component of the Rtt107-Slx4-Dpb11 complex. 4) The potential for additional binding partners for Dpb11, which may bind indirectly

via Rtt107 or Slx4. This is an especially strong possibility since our work further confirmed the critical scaffolding functions of Rtt107 by expanding its known protein interaction network to include Dpb11 and the Smc5-Smc6 structural maintenance of chromosomes complex (P. Ohouo, Bastos de Oliveira, Almeida, & Smolka, 2010). The relatively low cellular abundance of Dpb11 poses major limitation to our ability to detect such indirect binding partners. Studying the interaction network of Dpb11 under conditions where the number of molecules is increased and conducting a thorough analysis of the genetic interaction between the *slx4 S486A* allele and the different binding partners of Rtt107 and Slx4 may prove determinant when testing a model where the Rtt107-Slx4 complex enables the coordination of replication checkpoint signaling and repair (See figure 4.1).

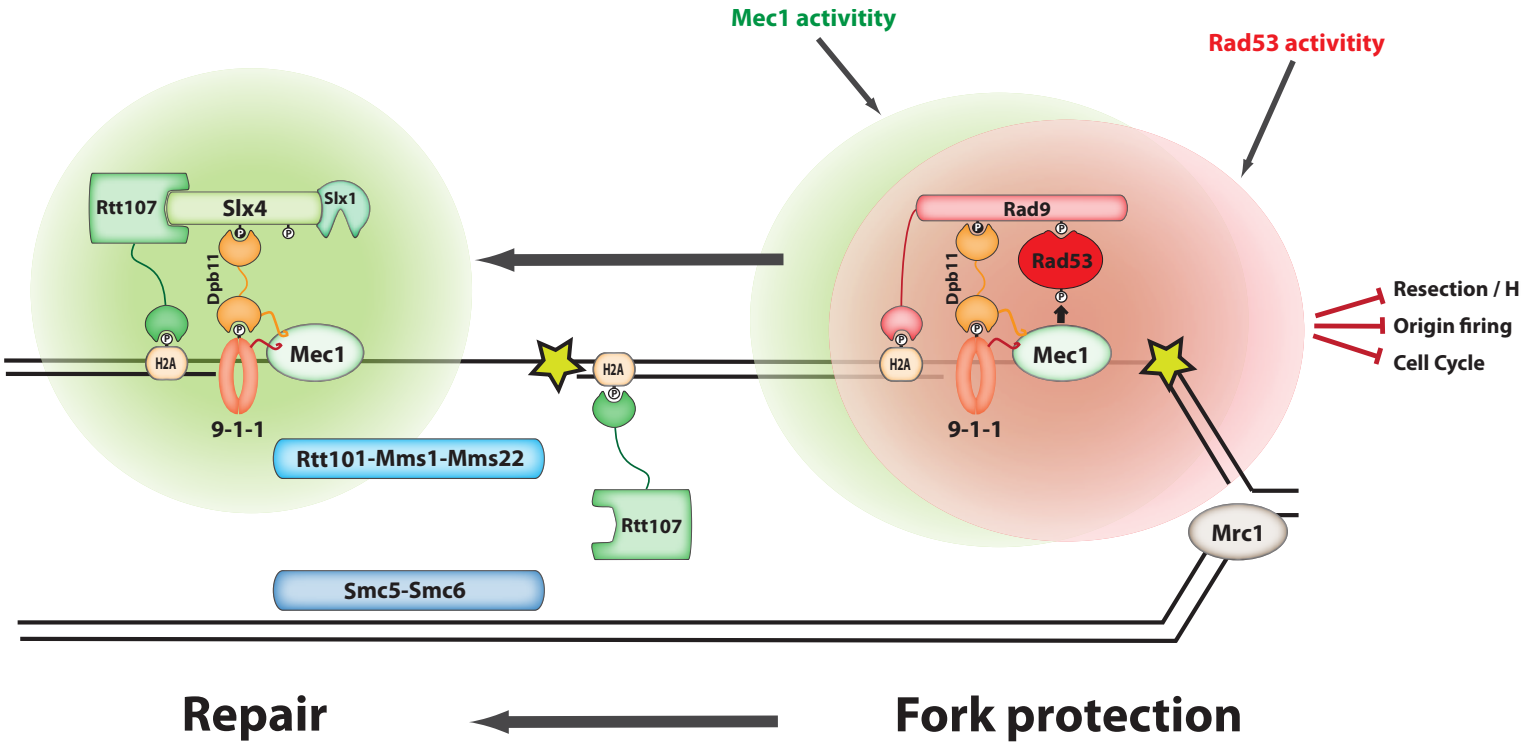

Mec1 activitity
Rad53 activitity
Rtt107
Slx4
Slx1
Dpb11
H2A
Mec1
9-1-1
Rtt101-Mms1-Mms22
Smc5-Smc6
Rtt107
Rad9
Rad53
Dpb11
H2A
Mec1
9-1-1
Mrc1
Resection / H
Origin firing
Cell Cycle
Repair
Fork protection

Figure 4-1

Figure 4.1. Model for the coordination of replication checkpoint signaling and DNA repair. Upon encountering lesion, replicative polymerase stalling leads to the accumulation of ssDNA behind the fork. RPA accumulation enables Mec1 recruitment. The 9-1-1 complex is recruited at the 5' ssDNA-dsDNA junction where it promotes the recruitment of Dpb11. H2A recruited near the site of damage is phosphorylated by Mec1 and mediates the recruitment of Rad9 leading to the activation of Rad53. γH2A mediated recruitment of Rtt107 may stabilizes the Rtt101 and Smc5-smc6 complexes at sites of damage to promote their repair functions.

FUTURE DIRECTIONS

My work has revealed the direct involvement of the replication checkpoint signaling, namely Mec1-dependent phosphorylation, in the functions of the Rtt107-Slx4 repair complex. Furthermore, I have identified a novel yet intricate mode for the downregulation of Rad53 mediated checkpoint signaling, which requires the recruitment of the Rtt107-Slx4 complex to the Dpb11 checkpoint protein. This mode of checkpoint regulation requires inputs from two distinct kinases, one which being Mec1. Several questions still need to be addressed, in order to further our understanding of the molecular mechanisms regulating the formation of the Rtt107-Slx4-Dpb11 complex and its biological relevance. First, what is the kinase responsible for the phosphorylation of Slx4 on S486, which is required for the formation of the Rtt107-Slx4-Dpb11 complex? Multisite phosphorylation regulated interactions were previously shown to enable the integration of different kinase pathways (Cohen, 2000). The identification of kinase responsible for S486 phosphorylation may therefore provide additional insights on the functional role of the Slx4-Dpb11 interaction. Second, how is the Mec1-dependent phosphorylation of Slx4 and phosphorylated S486 contributing to stabilize Slx4 onto Dpb11? Third, regarding the anti-checkpoint function of Slx4, what is the spatio-temporal dynamic of the formation of the Dpb11-Slx4 and Dpb11-Rad9 complexes? Finally, is the disruption of the anti-checkpoint function related to the aberrant chromosome structures common to *rtt107* and *slx4* null cells exposed to MMS? This latter question is especially relevant since deletion of

Slx1, an integral component of the Rtt107-Slx4-Dpb11 complex, does not lead to aberrant chromosome structures.

Identifying the kinase responsible for the phosphorylation of Slx4 on serine 486

The Rtt107-Slx4 complex and Dpb11 form a phosphorylation-dependent complex that is important for the downregulation of Rad53 and the preservation of viability during replication stress (P. Y. Ohouo, Bastos de Oliveira, Liu, Ma, & Smolka, 2013). Slx4, which bridges this interaction, is the target of two distinct phosphorylation events that are required for efficient Dpb11 binding (See figure 3-2). While we have identified the contribution of Mec1, which phosphorylates seven S/TQ motifs in the C terminus of Slx4, the kinase responsible for the direct phosphorylation of Slx4 on serine S486 remains to be determined. Three important indications make the Cyclin-Dependent Kinase (CDK) a very likely candidate. First, the S486 residue is followed by a proline, and is part of amino acid sequence that resembles the phosphorylation motif for CDK (Tak, Tanaka, Endo, Kamimura, & Araki, 2006). Second, CDK-dependent phosphorylation of known Dpb11 binding partners namely Sld2, Sld3 and Rad9, has been established as a requirement for their association with Dpb11 (Pfander & Diffley, 2011; Tak et al., 2006). Third, S486 aligns well with a proline-directed phosphorylation sites on Sld3, which is part of the Dpb11-binding region of Sld3 (see supplementary figure 3-6) (Zegerman & Diffley, 2010).

Investigating the respective mechanistic contribution of pS486 and the Mec1-dependent phosphorylation in the Slx4-Dpb11 interaction

We have identified two distinct phosphorylation events that mediate the binding between Slx4 and Dpb11. Based on our data, phosphorylated S486 (pS486) is required for the interaction where it mediates a low level of binding. On the other end, Mec1-dependent phosphorylation of seven canonical sites in the C-terminus of Slx4 is unable to mediate any detectable level of interaction in the absence of pS486 but appears to play an important role in stabilizing the complex. These observations suggest a two steps phosphorylation regulated model for the interaction where pS486 initially renders Slx4 competent to bind to Dpb11 and Mec1 subsequently strengthens the interaction. This view is strongly supported by the finding that in the absence of Rt1107, which is required for the Mec1-dependent phosphorylation of Slx4, Slx4 can still interact, although at a significantly lower level, with Dpb11. Similar intricate multi-steps phosphorylation regulated interactions have been documented for Dpb11 binding partners, namely Sld2 and Rad9 (Tak et al., 2006; Wang, Tong, Weng, & Zhou, 2012). In the case of Sld2, a critical threonine (Thr84) phosphorylated by CDK but not sufficient critical for Dpb11 binding. Several CDK-dependent phosphorylation events on nearby residues are in fact important for exposure and the efficient phosphorylation of Thr84 (Tak et al., 2006). In light of this observation, it is conceivable that the Mec1-dependent phosphorylation of Slx4 could induce a change of conformation in Slx4, which would enable efficient phosphorylation of S486 or expose additional interaction surfaces on Slx4. While

our work strongly suggest that pS486 plays direct roles in the interaction with Dpb11, it remains to be proven that it is an actual interaction interface. There may be additional point of interaction characterized by other CDK motifs, which may require Mec1-dependent phosphorylation in order to become exposed. The multiple BRCT domains of Dpb11 may recognize such additional interaction interfaces and help further stabilize the Slx4-Dpb11 complex. A thorough investigation of the interaction interfaces on Slx4 and Dpb11 is therefore critical to our understanding of the mechanistic contribution of Mec1 and pS486 in the formation of the Slx4-Dpb11 complex.

Finally, a two steps model for the regulation of the Slx4-Dpb11 interaction is particularly interesting in that it would ensure the timely recruitment of the checkpoint dampening functions of Slx4 only at sites were Rad53 activation is not longer beneficial.

Establishing the dynamics of the formation of the Dpb11-Slx4 and Dpb11-Rad9 complexes

The temporal aspect of the formation of the Dpb11-Slx4 and Dpb11-Rad9 complexes is an important component of our model on the anti-checkpoint function of Slx4 that remains to be established. While trying to understand the mechanisms regulating the association of Slx4 to Dpb11, we have made the observation that deletion of RAD53 leads to a significant decrease in the ability of the Rtt107-Slx4 complex to bind to Dpb11 (Figure 2.2e) (P. Ohouo et al., 2010). This suggests that Rad53 activation may promote the recruitment of the Rtt107-Slx4 complex to Dpb11. This is further supported by the fact that Rad53

extensively phosphorylates Dpb11 (Data not shown) and that Slx4 binds to the phosphorylated species of Dpb11 (Figure 2.2c) (P. Ohouo et al., 2010; P. Y. Ohouo et al., 2013). Such a requirement for Rad53 activation in the formation of the Dpb11-Slx4 complex could be assessed by using the *rad53 R605A* hypomorphic allele of Rad53, which significantly reduces the levels of Rad53 activation (P. Y. Ohouo et al., 2013). I expect to see a decrease in the Slx4-Dpb11 interaction in the *rad53 R605A* background when compared to wild type. An alternative approach would be to study the dynamics of Dpb11's interaction with Slx4 or Rad9. If Rad53 activation were proven to be required for Slx4 to bind Dpb11, it would temporally place the formation of the Dpb11-Rad9 complex before that of the Dpb11-Slx4 complex. Furthermore, it would suggest a negative feedback scenario where Rad53 can monitor and regulate its levels of activation. Ultimately, a global analysis of the relative localization of these two Dpb11-based complexes with respect to forks will be necessary to further test this model.

Investigating the link between chromosome abnormalities and the anti-checkpoint function of Slx4

Cells lacking *RTT107* or *SLX4* as opposed to *slx1Δ* cells, display excessive Rad53 activation along with persistent and aberrant DNA structures (Roberts et al., 2006). These DNA structures, suggested to result from incomplete DNA replication, point to the important role of Rtt107 and Slx4 in the resumption of DNA synthesis following replication fork stalling (Flott et al., 2007). While the nature of these DNA structures remains to be determined, our results on the anti-checkpoint functions of Rtt107 and Slx4 suggest that their persistence may

results from the hyperactivation of Rad53. This is further supported by the roles of Rad53 in inhibiting late origin firing or HR mediated repair (Alabert, Bianco, & Pasero, 2009; Zegerman & Diffley, 2010). It would be of great interest to assess the potential contribution of Rad53 in the formation of the persistent aberrant DNA structures observed in *rtt107Δ* or *slx4Δ* cells upon replication stress.

REFERENCES

Alabert, C., Bianco, J. N., & Pasero, P. (2009). Differential regulation of homologous recombination at DNA breaks and replication forks by the Mrc1 branch of the S-phase checkpoint. *EMBO J, 28*(8), 1131-1141. doi: 10.1038/emboj.2009.75

Cohen, P. (2000). The regulation of protein function by multisite phosphorylation--a 25 year update. *Trends Biochem Sci, 25*(12), 596-601.

Flott, S., Alabert, C., Toh, G., Toth, R., Sugawara, N., Campbell, D., . . . Rouse, J. (2007). Phosphorylation of Slx4 by Mec1 and Tel1 regulates the single-strand annealing mode of DNA repair in budding yeast. *Mol Cell Biol, 27*(18), 6433-6445.

Ohouo, P., Bastos de Oliveira, F., Almeida, B., & Smolka, M. (2010). DNA damage signaling recruits the Rtt107-Slx4 scaffolds via Dpb11 to mediate replication stress response. *Mol Cell, 39*(2), 300-306. doi: S1097-2765(10)00460-0 [pii]

10.1016/j.molcel.2010.06.019

Ohouo, P. Y., Bastos de Oliveira, F. M., Liu, Y., Ma, C. J., & Smolka, M. B. (2013). DNA-repair scaffolds dampen checkpoint signalling by counteracting the adaptor Rad9. N*ature, 493(*7430), 120-124. doi: 10.1038/nature11658

Pfander, B., & Diffley, J. F. (2011). Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment. E*MBO J, 30(*24), 4897-4907. doi: emboj2011345 [pii]

10.1038/emboj.2011.345

Roberts, T., Kobor, M., Bastin-Shanower, S., Ii, M., Horte, S., Gin, J., . . . Brown, G. (2006). Slx4 regulates DNA damage checkpoint-dependent phosphorylation of the BRCT domain protein Rtt107/Esc4. Mo*l Biol Cell, 17(1*), 539-548.

Tak, Y. S., Tanaka, Y., Endo, S., Kamimura, Y., & Araki, H. (2006). A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2-Dpb11. EM*BO J, 25(9*), 1987-1996. doi: 10.1038/sj.emboj.7601075

Wang, G., Tong, X., Weng, S., & Zhou, H. (2012). Multiple phosphorylation of Rad9 by CDK is required for DNA damage checkpoint activation. Ce*ll Cycle, 11(2*0), 3792-3800. doi: 10.4161/cc.21987

Zegerman, P., & Diffley, J. F. (2010). Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. Na*ture, 467(7*314), 474-478. doi: 10.1038/nature09373

APPENDIX

MATERIALS AND METHODS

Yeast strains and plasmids

Strains generated in this study were derived either from MBS164 or MBS191 (both congenic to S288C). Unless indicated, all tags were inserted at the C terminus of the corresponding genes by homologous recombination at the genomic loci and were verified by western blotting. Standard cloning methods were used to generate the plasmids for this study. SLX4 and DPB11 constructs containing an ADH1 promoter were generated by fusing the promoters (800 base pairs upstream of the start codon) to the corresponding open reading frame. The resultant PCR products were subsequently cloned into the pRS416 (Stratagene) or pFA6a (Addgene) vector. Wild-type alleles cloned into the pFA6a vector were linearized before integration into the respective endogenous loci. All point mutations were generated by site-directed mutagenesis using either the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) or the PFU Ultra II kit (Agilent). All yeast strains and plasmids used in this study are described in Tables A.1 and A.2 below.

Table A.1 Yeast strains used in this study

Strain No.	Relevant genotype
MBS164	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3*
MBS192	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::KanMX6*
MBS336	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2*
MBS373	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2, mrc1Δ:URA3, rtt107Δ:KanMX6*
MBS490	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, Smc6_6xHis-3xHA::KanMX6*
MBS494	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, Rtt101_6xHis-3xHA::KanMX6*
MBS574	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, Slx4_6xHis-3xFLAG::KanMX6*
MBS640	*ura3-52, trp1-63, his3-200*
MBS903	*MATa_ura3-52, trp1-63, his3-200*
MBS904	*MATa_ura3-52, trp1-63, his3-200, slx4Δ:HIS3*
MBS1780	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::NatMX4, rad9 S462A, S474A_6xHis-3xHA::KanMX6*
MBS1933	*MATa_ura3-52, trp1-63, his3-200, pADH1-Slx4_6xHis-3xFLAG::KanMX6*
MBS1935	*MATa_ura3-52, trp1-63, his3-200, pADH1-Slx4-7MUT_6xHis-3xFLAG::KanMX6*
MBS1937	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, RAD53_6xHis-3xHA::LEU2, pADH1-Slx4_6xHis-3xFLAG::KanMX6*
MBS1964	*MATa, ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1Δ:TRP1, bar1Δ:HIS3, ddc1Δ:KanMX6*

Table A.2 Plasmids used in this study

Plasmid No.	Vector	Gene	Mutation	Tag
pMBS148	pRS416	-	-	-
pMBS399	pFA6a	*rad9*	S462A, S474A	HA
pMBS414	pRS416	*pADH1-DPB11**	-	HA
pMBS433	pRS416	*pADH1-DPB11**	-	FLAG
pMBS439	pRS416	*pADH1-DPB11**	aa1-600	FLAG
pMBS426	pFA6a	*pADH1-SLX4**	-	FLAG
pMBS427	pFA6a	*pADH1-slx4**	T457A, T474A, S499A, T597A, S627A, S659A, S725A, S486A	FLAG

* Cloned with the ADH1 promoter.

Analysis of Slx4, Smc6 and Rtt101-Interacting Proteins by SILAC, HILIC and LC-MS/MS

The strains MBS164 (wild-type), MBS571 (Slx4-FLAG), MBS490 (Smc6-HA) or MBS494 (Rtt101-HA) were grown to O.D.600 of 0.4 in 400 mL of -Arg -Lys dropout media ("light" version complemented with normal arginine and lysine; "heavy" version complemented with L-Lysine 13C6, 15N2.HCl and L-Arginine 13C6, 15N4.HCl) and treated with 0.04% MMS for 2 hours. After centrifugation, pellets were kept at -80C prior to cell lysis. Approximately 0.6 g of cell pellet of each strain was lysed by bead beating at 4°C in 4 mL of lysis buffer (50 mM Tris-HCl pH 7.5, 0.2% Tergitol, 150 mM NaCl, 5 mM EDTA, Complete EDTA-free protease inhibitor cocktail (Roche), 5 mM sodium fluoride, 10 mM B-glycerol-phosphate). Lysate was incubated with anti-HA agarose resin (Sigma) for 4 hours at 4°C. After 3 washes with lysis buffer, bound proteins were eluted with 3 resin volumes of elution buffer (100 mM Tris-HCl pH 8.0, 1% SDS). Eluted proteins from normal or heavy media were mixed together, reduced, alkylated and then precipitated.

HILIC fractionation and mass spectrometry analysis

Proteins from pull-down experiments were solubilized with 200uL of 2M urea, 50mM Tris-HCL, pH 8.0, 150mM NaCl, followed by the addition of 2 ug of TPCK treated trypsin. Digestion was performed overnight at 37°C and then trifluoroacetic acid and formic acid were added to a final concentration of 0.2 %. Peptides were de-salted with Sep-Pak C18 column (Waters), dried in a speed-vac,

reconstituted in 85uL of a solution containing 80% acetonitrile and 1% formic acid and fractionated by Hidrophilic Interaction Chromatography (HILIC) as previously reported (Albuquerque et al., 2008). HILIC fractions were dried in a speed-vac, reconstituted in 0.1% trifluoroacetic acid and analyzed by LC-MS/MS using 125uM ID capillary C18 column and an Orbitrap XL mass spectrometer coupled with an Eksigent nano-flow system.

Database search and data analysis

Database search was performed using the Sorcerer system (Sagen) running Sequest program. After searching a target-decoy budding yeast database, results were filter based on Probability Score to achieve a 1 % false positive rate. Quantitation of heavy / light peptide isotope ratios was performed using the Xpress program as previously described (Smolka, Albuquerque, Chen, & Zhou, 2007). All results were also manually inspected.

Cell synchronization and genotoxin treatment

Yeast cells were grown in yeast peptone dextrose (YPD) or drop-out medium at 30 °C. Log phase cultures (optical density at 600 nm ≈ 0.3) were subjected to α-factor (0.5 μg ml^{-1}) for G1 arrest. Cells were then washed and resuspended in warm medium containing the indicated genotoxin.

Cell lysis and Western blotting

For western blotting, about 50 mg frozen cell pellet was lysed by bead beating at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.2% Tergitol, 150 mM

NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), Complete, EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP (Roche)). SDS loading buffer with 60 mM dithiothreitol (DTT) was added. Samples were separated by standard SDS-polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were detected using the following antibodies: anti-HA (12CA5, 1:10,000, Roche) and anti-Myc (SC-40, 1:5000, Santa-Cruz) antibodies.

Cell collection and FACS analysis

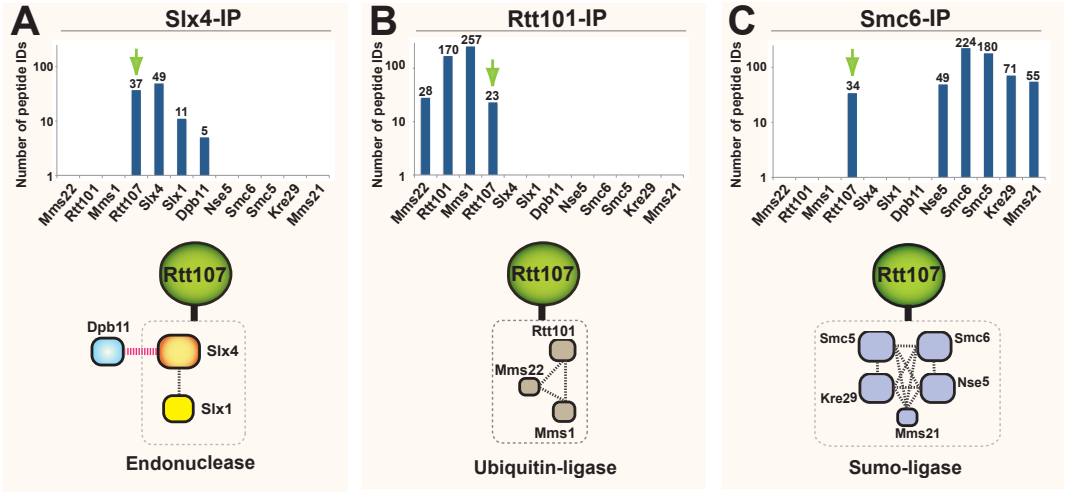
Cells were grown in liquid YPD to O.D= 0.2 before addition of α-factor for 2hrs. G1 samples were collected and remaining cultures were washed in warm YPD before release in YPD with 0.01% MMS. Samples collected at indicated time points were fixed in 70% ethanol at 4°C. The fixed cells were dryed, resuspended in 50 mM sodium citrate, treated with 0.2 mg/ml RNase A (Qiagen) at 37°C for 1h, and then with 0.5 mg/ml proteinase K (Invotrogen) at 42°C for 1h. Cells were then stained for 2hrs in 5nM Sytox green (Invitrogen) and at 4°C. The DNA content of cells was analyzed with a BD Accuri C6 flow cytometer.

Investigating the contribution of Mec1 in the repair functions of Rtt107

Our detailed analysis of the protein interaction network of Rtt1017 showed that it is part of mutually exclusive complexes with the Rtt101-based Ubiquitin-Ligase complex (Rtt101-Mms1-Mms22), the Smc5-Smc6 Sumo-Ligase complex (Smc5, Smc6, Nse1, Nse3-6) and the Dpb11-Slx4-Slx1 complex (Figure A.1a, b and c). These different complexes point to the involvement of Rtt107 in different repair pathways (Figure A.1d). The extensive phosphorylation of Rtt107 by Mec1, which initially suggested that Mec1 directly regulates its scaffolding functions, seems so far to be coincidental to Rtt107's involvement in the Dpb11-Slx4 pathway (P. Ohouo, Bastos de Oliveira, Almeida, & Smolka, 2010). Nonetheless, our work and others show that Mec1 may significantly contribute to the different repair functions of Rtt107 through the phosphorylation of H2A, which appears to be essential in mediating the recruitment of Rtt107 to sites of damage (Li et al., 2012; P. Y. Ohouo, Bastos de Oliveira, Liu, Ma, & Smolka, 2013). This is illustrated by the fact that the disruption of the Rtt107-γH2A interaction leads to HU, CPT or MMS sensitivity similar to that of *rtt107Δ* cells (Li et al., 2012).

In sum, Rtt107 represents a mechanistic link between DNA repair and replication checkpoint signaling and may help coordinate these two pathways. On one hand, Rtt107 may promote the recruitment of Rtt101 and the Smc5-Smc6 complexes and other key repair factors to sites of damage in a checkpoint dependent manner via γH2A. On the other, it may enable the concomitant and

timely down regulation of Rad53 to promote HR and late origin firing (See speculative model: Figure 4.1).


A
Slx4-IP
Number of peptide IDs
100
10
1
37
49
11
5
Mms22 Rtt101 Mms1 Rtt107 Slx4 Slx1 Dpb11 Nse5 Smc6 Smc5 Kre29 Mms21
Rtt107
Dpb11
Slx4
Slx1
Endonuclease
B
Rtt101-IP
Number of peptide IDs
100
10
1
28
170
257
23
Mms22 Rtt101 Mms1 Rtt107 Slx4 Slx1 Dpb11 Nse5 Smc6 Smc5 Kre29 Mms21
Rtt107
Rtt101
Mms22
Mms1
Ubiquitin-ligase
C
Smc6-IP
Number of peptide IDs
100
10
1
34
49
224
180
71
55
Mms22 Rtt101 Mms1 Rtt107 Slx4 Slx1 Dpb11 Nse5 Smc6 Smc5 Kre29 Mms21
Rtt107
Smc5
Smc6
Kre29
Nse5
Mms21
Sumo-ligase

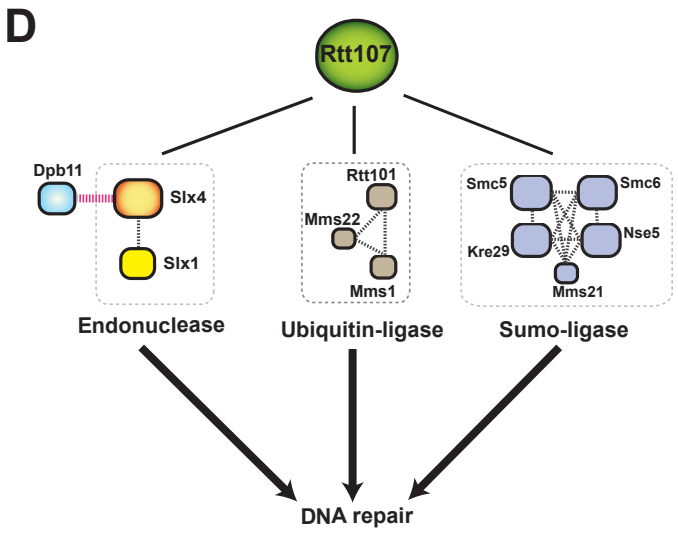

D
Rtt107
Dpb11
Slx4
Slx1
Endonuclease
Rtt101
Mms22
Mms1
Ubiquitin-ligase
Smc5
Smc6
Kre29
Nse5
Mms21
Sumo-ligase
DNA repair

Figure A-1

Figure A.1. Rtt107 forms mutually exclusive complexes that contribute to repair. (a), (b) and (c) Mass spec analysis of Slx4, Smc6 and Rtt101 pull downs (bait proteins indicated by green arrow). The numbers above the bars refer to the number of peptides detected for each indicated binding partner. Slx4 was Flag tagged at the C-terminus. Smc6 and Rtt101 were HA tagged at the C-terminus. (d) Model: Rtt07 promotes repair through its physical interaction with different repair complexes.

A Dpb11 overexpression system to further test the anti-checkpoint function of Slx4

The checkpoint functions of Dpb11 involve the stimulation of the kinase activity of Mec1 and the promotion of Rad53 activation in a Rad9-dependent manner (Pfander & Diffley, 2011). In order to promote Mec1 activation, Dpb11 needs to form a complex with Mec1 and Ddc2 (human ATRIP) (Mordes, Nam, & Cortez, 2008). Mutations in Ddc2 (*ddc2-top*) that disrupt the Ddc2-Dpb11 interaction lead to checkpoint activation defects (Mordes et al., 2008). Remarkably, overexpression of Dpb11 was shown to correct the checkpoint defects associated with the *ddc2-top* mutant allele (Mordes et al., 2008). Although this finding was mainly explained by the ability of Dpb11 to stimulate the kinase activity of Mec1, it does not exclude the possibility for Dpb11 overexpression to lead to the hyperactivation of the Rad9-Rad53 pathway. In order to further delineate the repair functions associated with the Rtt107-Slx4 complex from its newly characterized anti-checkpoint functions, I decided to use a similar Dpb11 overexpression system. My hypotheses were that 1) overexpression of Dpb11 should lead to Rad9-dependent Rad53 hyperactivation and sensitivity to MMS. 2) Co-overexpression of Slx4 should reverse the phenotypes observed upon Dpb11 overexpression.

In an effort to better appreciate the contribution of Dpb11 mediated activation of Mec1 in this overexpression system; I chose to use a truncation of Dpb11, *dpb11ΔC* (amino acid 1 to 600) as a control. This mutant lacks the C-terminal domain of Dpb11, which is necessary to stimulate Mec1 activity (Mordes

et al., 2008; Navadgi-Patil & Burgers, 2008). I found that as opposed to empty vector, overexpression of full length Dpb11 leads to hypersensitivity to MMS (Figure A.2a). Overexpression of *dpb11ΔC* leads to a level of MMS sensitivity that is less than that of full length Dpb11 (Figure A.2a). Interestingly, the MMS sensitivities observed correlate with the levels of Rad53 activation, which were significantly higher in cells overexpressing full length Dpb11 (Figure A.2b). FACS analysis shows that as opposed to control, Dpb11 overexpressing progress significantly slower through the cell cycle only in the presence of MMS (Figure A.2c), which correlates with the hypersensitivity of these cells to MMS.

Although the interpretation of this result is rendered difficult by the tight connection between the kinase activity of Mec1 and Rad9's ability to promote Rad53 activation (Pfander & Diffley, 2011; Schwartz et al., 2002; Sun, Hsiao, Fay, & Stern, 1998), the data presented here strongly suggests that the cellular sensitivity to MMS of Dpb11 overexpressing cells results from excessive activation of Rad53. Interestingly, this excessive Rad53 activation is comparable to the one observed in *slx4Δ* cells (P. Y. Ohouo et al., 2013).

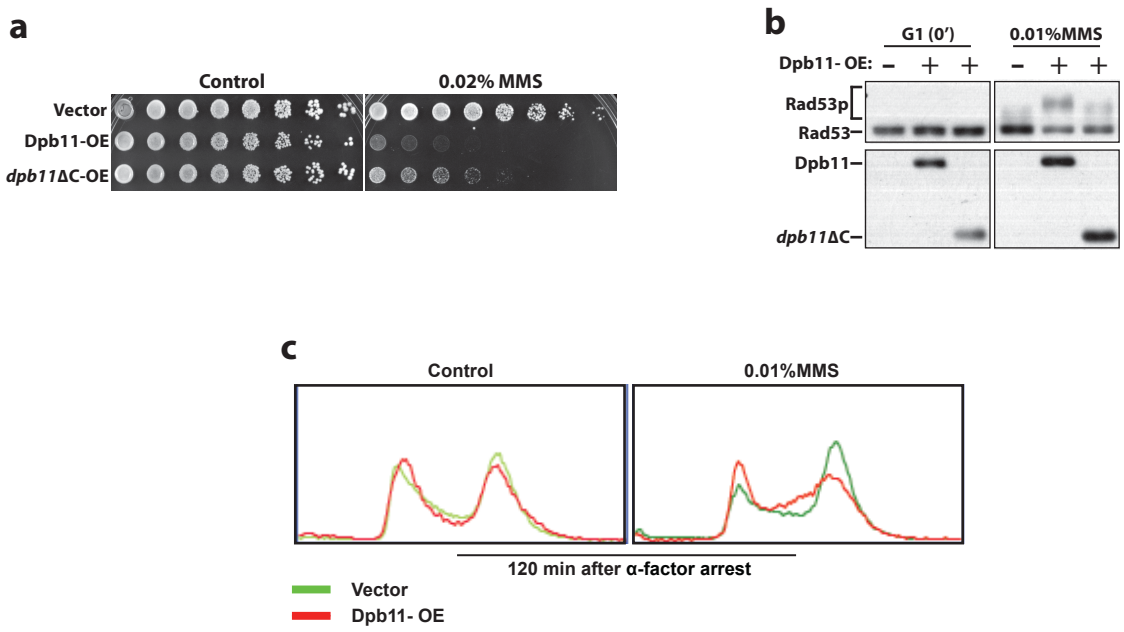

a
Control
0.02% MMS
Vector
Dpb11-OE
dpb11ΔC-OE
b
G1 (0')
0.01%MMS
Dpb11- OE: – + + – + +
Rad53p
Rad53
Dpb11
dpb11ΔC
c
Control
0.01%MMS
120 min after α-factor arrest
Vector
Dpb11- OE

Figure A-2

Figure A.2. Overexpression of Dpb11 leads to hypersensitivity to MMS and excessive Rad53 activation. (a) MMS sensitivity of Dpb11 or *dpb11ΔC* overexpressing cells. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30°C. (b) Western blot analysis showing the phosphorylation status of HA tagged Rad53 in Dpb11 or *dpb11ΔC* overexpressing cells. C-terminal FLAG tagged Dpb11 or *dpb11ΔC* (lower panel) are expressed ectopically under the control of the ADH1 promoter. (c) FACS analysis comparing the DNA content from cells with empty vector (green) and overexpressing full length Dpb11 (red) in the presence or absence of MMS. C-terminal FLAG tagged Dpb11 was expressed ectopically under the control of the ADH1 promoter.

In trying to understand the molecular mechanisms underlying the observations made above, I conducted similar sensitivity experiments in different mutant backgrounds. I found that the deletion of the checkpoint clamp component Ddc1 leads to the rescue of the MMS sensitivity of Dpb11 overexpressing cells (Figure A.3a). Ddc1 is critical for the recruitment of Dpb11 at 5' ssDNA-dsDNA (Ellison & Stillman, 2003; Majka, Binz, Wold, & Burgers, 2006). Consequently, the rescue observed here suggests that the MMS sensitivity resulting from Dpb11 overexpression is specific to the checkpoint function of Dpb11. To home in better on whether the overexpression of Dpb11 potentiated the Rad9-Rad53 pathway, I made used of a recently characterized hypomorphic allele of Rad9, where mutations of serine 462 (S462) and serine 474 (S474) to alanine was shown to disrupt the ability of Rad9 to bind to Dpb11 and enable robust Rad9-dependent Rad53 activation (Pfander & Diffley, 2011). I found that overexpression of full length Dpb11 in the *rad9 S462A, S474A* background leads to a significantly lower level of MMS sensitivity when compared to wild type cells (Figure A.3b). Altogether, these findings indicate that Dpb11 overexpression leads to the hyperactivation of Rad53 in a Rad9-dependent manner. Furthermore, the MMS sensitivity resulting from Dpb11 overexpression appears to result from excessive Rad53 activation, which would be similar to what is observed in cells lacking SLX4 (P. Y. Ohouo et al., 2013). In sum, these results validate our Dpb11 overexpression system with respect to testing the anti-checkpoint functions of Slx4.

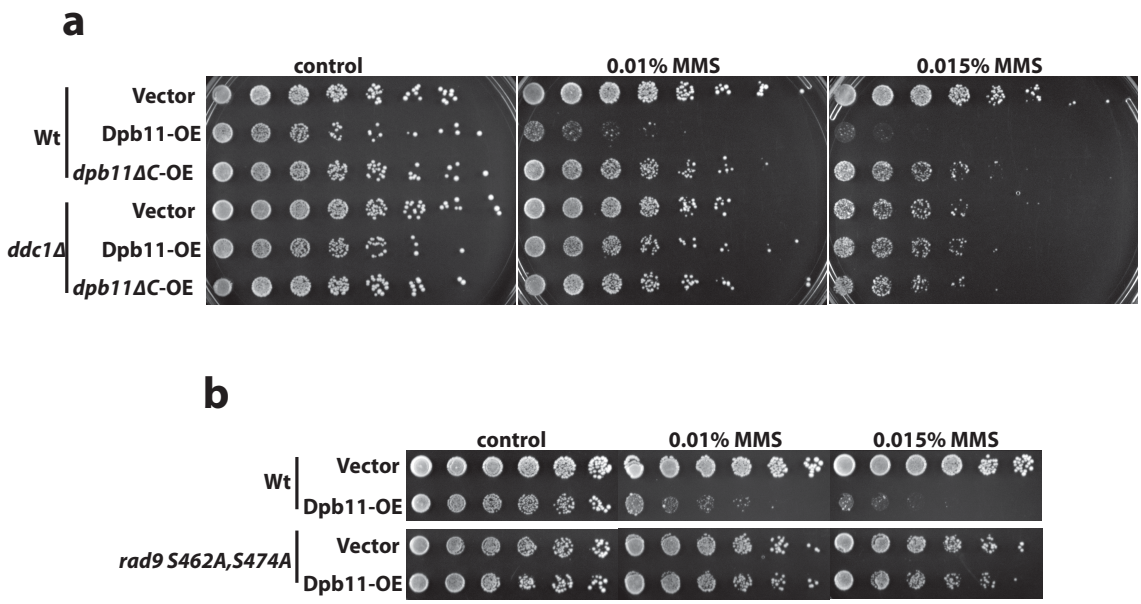

a
control
0.01% MMS
0.015% MMS
Wt
Vector
Dpb11-OE
dpb11ΔC-OE
ddc1Δ
Vector
Dpb11-OE
dpb11ΔC-OE
b
control
0.01% MMS
0.015% MMS
Wt
Vector
Dpb11-OE
rad9 S462A,S474A
Vector
Dpb11-OE

Figure A-3

Figure A.3. Overexpression of Dpb11 leads to hypersensitivity in a Ddc1 and Rad9-dependent manner. (a) MMS sensitivity of Dpb11 or *dpb11ΔC* overexpressing cells in wild type (Wt) or *ddc1Δ* background. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30°C. (b) MMS sensitivity of Dpb11 overexpressing cells in wild type (Wt) or *rad9 S462A, S474A* background. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30°C. C-terminal FLAG tagged Dpb11 or *dpb11ΔC* (lower panel) are expressed ectopically under the control of the ADH1 promoter. HA tagged *rad9 S462A, S474A* was integrated at the *RAD9* locus and expressed under endogenous promoter.

The Dpb11 overexpression system developed here provides a tool to study the anti-checkpoint function of Slx4 without resorting to the use of disruptive mutations that may impair its repair functions. I hypothesized that if Slx4 counteracts the Rad9-Rad53 pathway at the level of Dpb11, co-overexpression of Slx4 with Dpb11 should rescue the MMS sensitivity and decrease the level Rad53 activation observed upon Dpb11 overexpression. We found that indeed, co-overexpression of Slx4 with Dpb11 significantly rescues the MMS sensitivity observed upon Dpb11 overexpression alone (Figure A.4a). This is specific to the ability of Slx4 to bind Dpb11 because co-overexpression of *slx4-7MUT* leads to a rescue lesser than that of wild type Slx4 (Figure A.4a). Most importantly, co-overexpression of Slx4 and Dpb11 significantly reduces Rad53 hyperactivation (Figure A.4b). These findings strongly support a model where Slx4 binding to Dpb11 counteracts the Rad9-Rad53 pathway.

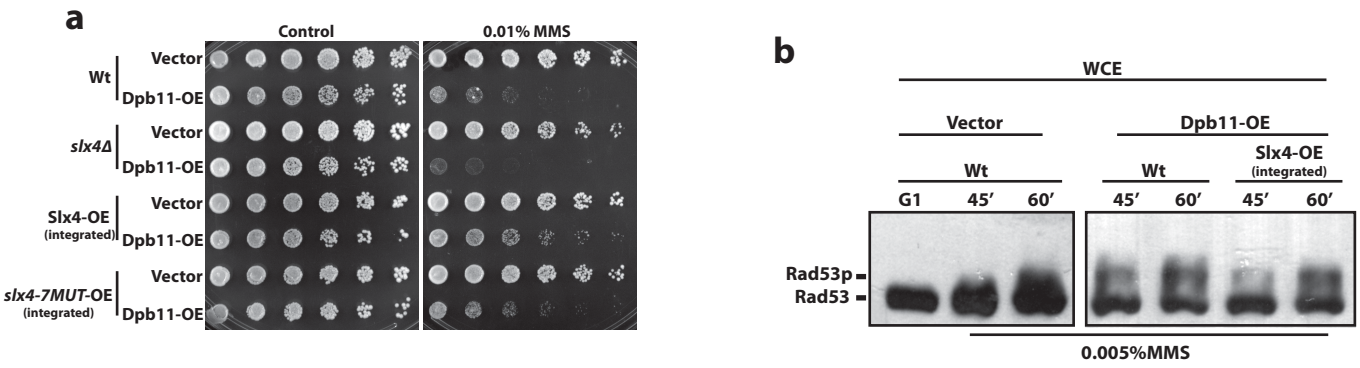

a
Control
0.01% MMS
Wt
Vector
Dpb11-OE
slx4Δ
Vector
Dpb11-OE
Slx4-OE
(integrated)
Vector
Dpb11-OE
slx4-7MUT-OE
(integrated)
Vector
Dpb11-OE
b
WCE
Vector
Dpb11-OE
Wt
Wt
Slx4-OE
(integrated)
G1
45′
60′
45′
60′
45′
60′
Rad53p
Rad53
0.005%MMS

Figure A-4

Figure A.4. Co-overexpression of Slx4 with Dpb11 reduces the MMS sensitivity and excessive Rad53 activation resulting form Dpb11 overexpression. (a) MMS sensitivity of Dpb11 overexpressing cells in Wt or *slx4-7MUT* background. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30°C. (b) Western blot analysis showing the phosphorylation status of HA tagged Rad53 in Dpb11 overexpressing or Dpb11 and Slx4 co-overexpressing cells. C-terminal FLAG tagged Dpb11 was expressed ectopically under the control of the ADH1 promoter. C-terminal Myc tagged Slx4 or *slx4-7MUT* were overexpressed from the SLX4 endogenous locus under the control of the ADH1 promoter.

REFERENCES

Alabert, C., Bianco, J. N., & Pasero, P. (2009). Differential regulation of homologous recombination at DNA breaks and replication forks by the Mrc1 branch of the S-phase checkpoint. *EMBO J, 28*(8), 1131-1141. doi: 10.1038/emboj.2009.75

Ellison, V., & Stillman, B. (2003). Biochemical characterization of DNA damage checkpoint complexes: clamp loader and clamp complexes with specificity for 5' recessed DNA. *PLoS Biol, 1*(2), E33. doi: 10.1371/journal.pbio.0000033

Flott, S., Alabert, C., Toh, G., Toth, R., Sugawara, N., Campbell, D., . . . Rouse, J. (2007). Phosphorylation of Slx4 by Mec1 and Tel1 regulates the single-strand annealing mode of DNA repair in budding yeast. *Mol Cell Biol, 27*(18), 6433-6445.

Li, X., Liu, K., Li, F., Wang, J., Huang, H., Wu, J., & Shi, Y. (2012). Structure of C-terminal tandem BRCT repeats of Rtt107 protein reveals critical role in interaction with phosphorylated histone H2A during DNA damage repair. *J Biol Chem, 287*(12), 9137-9146. doi: 10.1074/jbc.M111.311860

Majka, J., Binz, S. K., Wold, M. S., & Burgers, P. M. (2006). Replication protein A directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions. *J Biol Chem, 281*(38), 27855-27861. doi: 10.1074/jbc.M605176200

Mordes, D., Nam, E., & Cortez, D. (2008). Dpb11 activates the Mec1-Ddc2 complex. *Proc Natl Acad Sci U S A, 105*(48), 18730-18734. doi: 0806621105 [pii] 10.1073/pnas.0806621105

Navadgi-Patil, V., & Burgers, P. (2008). Yeast DNA replication protein Dpb11 activates the Mec1/ATR checkpoint kinase. J *Biol Chem, 283(*51), 35853-35859. doi: M807435200 [pii]

10.1074/jbc.M807435200

Ohouo, P., Bastos de Oliveira, F., Almeida, B., & Smolka, M. (2010). DNA damage signaling recruits the Rtt107-Slx4 scaffolds via Dpb11 to mediate replication stress response. Mo*l Cell, 39(2*), 300-306. doi: S1097-2765(10)00460-0 [pii]

10.1016/j.molcel.2010.06.019

Ohouo, P. Y., Bastos de Oliveira, F. M., Liu, Y., Ma, C. J., & Smolka, M. B. (2013). DNA-repair scaffolds dampen checkpoint signalling by counteracting the adaptor Rad9. Nat*ure, 493(74*30), 120-124. doi: 10.1038/nature11658

Pfander, B., & Diffley, J. F. (2011). Dpb11 coordinates Mec1 kinase activation with cell cycle-regulated Rad9 recruitment. EMB*O J, 30(24*), 4897-4907. doi: emboj2011345 [pii]

10.1038/emboj.2011.345

Schwartz, M. F., Duong, J. K., Sun, Z., Morrow, J. S., Pradhan, D., & Stern, D. F. (2002). Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint. Mol *Cell, 9(5),* 1055-1065.

Smolka, M., Albuquerque, C., Chen, S., & Zhou, H. (2007). Proteome-wide identification of in vivo targets of DNA damage checkpoint kinases. Proc *Natl Acad Sci U S A, 104(25)*, 10364-10369.

Sun, Z., Hsiao, J., Fay, D. S., & Stern, D. F. (1998). Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Scie*nce, 281(537*4), 272-274.

Zegerman, P., & Diffley, J. F. (2010). Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. Natu*re, 467(731*4), 474-478. doi: 10.1038/nature09373